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The Distribution of Adenosine Triphosphate in Subcellular Fractions of Brain Tissue

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Most of the bound acetylcholine (Whittaker, 1959), hydroxytryptamine (Whittaker, 1959; Michaelson & Whittaker, 1962) and noradrenaline (Chruściel, 1960) of brain tissue are recovered in a subcellular fraction which consists mainly of pinched-off nerve endings (Gray & Whittaker, 1960, 1962). The work now to be described originated in an attempt to see whether ATP was involved in the binding of the pressor amines to the particles of this fraction, as it seems to be in the denser and morphologically different type of storage granule found in adrenal medulla, platelets, duodenal mucosa and adrenergic nerves (for reviews see Hagen & Barrnett, 1960; Blaschko, 1959).

A brief account of this work has been given by Whittaker (1961).

METHODS

Preparation of subcellular fractions from brain tissue. Fractions were prepared at 0–4° from guinea-pig and dog

brain essentially as described by Whittaker (1959) and Gray & Whittaker (1962) and as summarized in Scheme 1. Unless otherwise stated adult guinea pigs (400–500 g. body wt.) were killed by decapitation, with or without a preceding blow at the base of the skull. Dogs were killed under ether or nembutal anaesthesia by bleeding or by sectioning the brain stem. The brains were removed as rapidly as possible and chilled before dissection and homogenization. Factors making for satisfactory recoveries were as follows. (1) All traces of detergent were excluded from the apparatus used in the experiment, otherwise losses of ATP of up to 90% could occur. Glassware was cleaned by brushing in hot water and rinsing in dilute HCl, followed by glass-distilled water. (2) Adequate refrigeration was maintained, especially during centrifuging. The use of the Servall refrigerated automatic centrifuge type SS-34 in place of the bench model (SS-1) in the cold room for the preparation of the P_2 fraction doubled the ATP content of this fraction. Density-gradient separations at 39 000 rev./min. for 45 min. instead of at 25 000 rev./min. for 2 hr. increased the recovery of ATP in the A, B and C subfractions by over 25%. (3) Pellets were resuspended with as little mechanical disturbance as possible. Even so, 15–20% of 'stable' ATP was lost during the preparation of P_2 , and a further 10% during high-speed centrifuging.

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A spectroscopic determination of the haemoglobin content of the red cells present in the initial homogenate, which sediment at the bottom of the P_1 pellet, indicated a blood content of the brain tissue of less than 0.5% (v/w). With an ATP content of blood of about 100 $\mu\text{g./ml.}$, the contribution from blood to the total ATP content of the homogenate was estimated to be less than 0.25% (less than the error of the estimation). Experiments in which enough guinea-pig blood was added to the initial homogenate to render it distinctly pink also showed no increase in the ATP content of the fractions.

Extraction of ATP from brain homogenates and sub-cellular fractions. Three ways of extracting ATP from preparations were compared (Table 1). Extraction with trichloroacetic acid in a final concentration of 10% (w/v) gave the highest values and these were unaffected by prior homogenization in 0.32M-sucrose. The supernatant from trichloroacetic acid after ether extraction was between pH 4

and 5. Neutralization with NaOH at this stage did not increase the ATP values found and could lead to losses owing to the extreme lability of ATP in these concentrations above pH 7.5.

Estimation of ATP. ATP was estimated by the firefly-luminescence method, essentially as described by Chase (1960). Dried firefly lanterns (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.) were homogenized (100 mg./ml.) in 0.1M-sodium arsenate at pH 8.0. After centrifuging, the supernatant was stored at -20° in small tubes. This was thawed and diluted with ice-cold glass-distilled water immediately before use and was stable at 0° for 6-8 hr. The reaction mixture consisted of 0.9 ml. of 0.05M-glycylglycine buffer, pH 7.6, 0.1 ml. of 0.1M-MgCl₂, 0.1 ml. of enzyme (equivalent to 1-2 mg. of dried lanterns) and 0.2 ml. of sample. The last two constituents were added while still ice-cold to the first two at room temperature, and, 15 sec. later, the light produced was measured in the Locarte model

Scheme 1. Separation of fractions and identification of symbols used in text and Tables.

Table 1. Comparison of methods for extraction of adenosine triphosphate from guinea-pig brain

All operations were carried out at $0-4^\circ$ with ice-cold reagents. Samples were stored at -20° immediately after treatment until estimated.

Treatment	Concn. of ATP ($\mu\text{g./g. wet wt.}$)	Percentage of (a)
(a) Tissue homogenized in 10% (w/v) trichloroacetic acid and centrifuged; supernatant extracted with ether; ether removed with N_2	190	—
(b) Tissue homogenized in 0.32M-sucrose, treated with an equal volume of 20% (w/v) trichloroacetic acid and then treated as (a)	200	105
(c) Tissue homogenized in 0.32M-sucrose; perchloric acid added to 6% (w/v) final concn.; neutralized with KOH and centrifuged; precipitate discarded	124	65
(d) Tissue homogenized in 0.32M-sucrose and adjusted to pH 4; heated at 100° for 10 min., chilled and neutralized	114	60

LFM/4 fluorimeter with the light-source switched off and the photomultiplier near its maximum setting. Weights of ATP refer to the ATP anion. The error of the method was estimated at $\pm 5\%$.

The photomultiplier output as measured by the galvanometer showed a linear relation to ATP concentration in the range 0.5–4 $\mu\text{g.}$ of ATP/ml., which corresponds to the amount of ATP in 2.5–20 mg. of whole brain. Extracts were normally diluted at least 10-fold before estimation and then contained no more than the equivalent of 10 mg. of tissue and 80 μmoles of sucrose/ml. Under these conditions there was no interference with the amount of light emitted for a given amount of ATP as compared with controls, and the recovery of added ATP was 100%. Co-enzyme A (up to 300 μmoles /ml., i.e. in amounts greatly in excess of those likely to be present in the extracts) gave no inhibition.

Total nitrogen. This was determined by the micro-Kjeldahl method.

RESULTS

Post-mortem changes in the ATP content of whole guinea-pig brain

At the outset of the work it was realized that the rapid fall that occurs *post mortem* in the amount of brain ATP (for references see McIlwain, 1959) would place certain limitations on any attempt to study the regional or subcellular distribution of this substance. It was therefore decided to determine the extent of the fall in guinea-pig brain and the relationship of the ATP content of homogenates of brain in 0.32M-sucrose to total brain ATP at different times after death.

To follow the post-mortem changes in brain ATP young guinea pigs weighing 130–150 g. were selected. One animal (Table 2, col. 2) was killed by rapid total immersion in liquid nitrogen. The brain was chiselled out and pulverized under liquid nitrogen. The ensuing powder was extracted while still frozen with ice-cold 10% trichloroacetic acid in a homogenizer. The ATP value obtained from this extract is assumed to represent the instantaneous post-mortem value. A second animal (Table 2,

col. 3) was killed by decapitation, the brain dissected out as rapidly as possible and one hemisphere homogenized in 10% (w/v) ice-cold trichloroacetic acid, a process completed in 1 min. The other hemisphere was kept at 4° for 45 min. before being similarly homogenized. A third animal (Table 2, col. 4) was similarly treated except that homogenization of the two hemispheres was completed at 5 and 60 min. after decapitation respectively.

The amounts of ATP found in these extracts are presented in Table 2. The ATP concentration in the brain of the animal killed by immersion in liquid nitrogen was about 4 $\mu\text{moles/g.}$ wet wt., which is in accordance with the values cited by McIlwain (1959). The other figures confirm the statement by McIlwain (1959) that 90% of the brain ATP disappears during the first few minutes after death. But it is also clear from Table 2 that the residual 10% left after these first few minutes is metabolically much more stable than the main fraction and remains unchanged at least up to 1 hr. *post mortem*. This relatively stable fraction of ATP survives homogenization in sucrose, as was shown in experiments in which the ATP content of one hemisphere, determined by homogenizing the tissue in trichloroacetic acid, was compared with that of the other, determined on a homogenate in 0.32M-sucrose. Entries (a) and (b) in Table 1 constitute one such experiment.

Sucrose homogenates could be kept for up to 6 hr. at 0° before there was an appreciable fall in their ATP content. This is shown in Table 2 (col. 5). It is the regional and subcellular distribution of this 'stable' fraction of ATP that is the subject of the rest of this paper.

The 'stable' ATP of adult guinea-pig brain was found to be remarkably constant (mean of 15 determinations, 210, s.d. $\pm 21 \mu\text{g./g.}$ wet wt.). The corresponding values for younger animals were higher, which accounts for the difference between cols. 2–4 and col. 5 in Table 2.

Table 2. *Post-mortem changes in the content of adenosine triphosphate of guinea-pig brain at 0–4°*

Time after death (min.)	Concn. of ATP ($\mu\text{g./g.}$ wet wt.)		
	Killed in liquid N ₂ *	Homogenized in 10% (w/v) trichloroacetic acid*	Homogenized in sucrose†
0	2120	—	—
1	—	650	—
5	—	—	330
45	—	380	—
60	—	—	330
150	—	—	210
240	—	—	205
360	—	—	210
480	—	—	197
			148

* Young animals.

† Adult animal.

Distribution of 'stable' ATP in different regions of the brain

As is shown in Table 3, 'stable' ATP seems to be distributed fairly uniformly throughout the whole brain and to be present in both grey and white matter (internal capsule, cerebellar white matter). The amounts of ATP in pure white matter were, however, less reproducible than those in other structures. In guinea pigs this might have been partly accounted for by technical difficulties associated with the small samples, but the variation in dog-brain white matter, where the samples were much larger, cannot be accounted for in this way and seems to indicate that the 'stable' ATP fraction is more labile in white matter than in grey. The low figures in the first dog experiment in which the animal was killed for another purpose by exsanguination were probably due to the delay (over 20 min.) between death and dissection.

Subcellular distribution of 'stable' ATP

Distribution in fractions from whole brain. Homogenization of brain tissue leads to the production of five main kinds of subcellular particles: nuclei, large and small myelin fragments, torn-off nerve endings, mitochondria and microsomes (Gray & Whittaker, 1962). The procedure of fractionation shown in Scheme 1, although not completely separating all these particles, enables nerve endings, mitochondria and part of the myelin to be obtained in fairly homogeneous form.

The distribution of 'stable' ATP in these fractions is given in Table 4. It will be seen that 26-50 % of the 'stable' ATP is lost during the whole fractionation. Of the 50-74 % recovered, about half is found in the nerve-ending fraction (B); of the remainder, about 20 % is recovered in fraction P₁, 20 % in the combined microsomal and soluble supernatant fraction (S₂) and about 10 % fairly

Table 3. Distribution of 'stable' adenosine triphosphate in different regions of guinea-pig and dog brain

Concn. of ATP (μg./g. wet wt.)							
Cerebral cortex	Internal capsule	Caudate nucleus	Thalamus	Hypothalamus	Pons	Cerebellum	Medulla oblongata
Guinea pig							
150	50	187	255	210	142	250	187
185	187	229	195	187	—	202	155
240	396	—	—	—	—	—	—
Dog							
						Grey	White
110	95.5	125	130	100	—	100	125
176	80	—	—	—	—	—	—
340	280	—	—	—	—	—	—
350	165	—	—	—	—	—	—

Table 4. Distribution of 'stable' adenosine triphosphate in subcellular fractions of guinea-pig brain

The concentration of ATP in each fraction (symbols as in Scheme 1) is given as μg./vol. of fraction equivalent to 1 g. of brain and (in parentheses) as a percentage of the total recovered ATP. In the first five experiments, the supernatant S₂ from the centrifuging carried out at 17 000 g was not separated into P₂ and S₂.

H	P ₁	P ₂	S ₂	P ₃	S ₃	Recovery in primary fractions (as % of H)	A	B	C	Recovery in sub-fractions (as % of P ₂)	Overall recovery (as % of H)
209	32 (15)	97 (70)	27 (14)	—	—	75	—	—	—	—	—
187	32 (18)	104 (60)	36 (27)	—	—	93	—	—	—	—	—
240	34 (17)	117 (62)	42 (21)	—	—	82	—	—	—	—	—
189	16 (11)	93 (64)	37 (25)	—	—	77	19 (22)	56 (69)	8 (9)	89	72
200	31 (20)	95 (61)	27 (18)	—	—	76	17 (19)	61 (69)	11 (12)	91	74
Mean											
205	29 (16)	101 (63)	34 (21)	—	—	81	—	—	—	—	—
207	24 (21)	85 (71)	—	2 (1)	9 (7)	60	—	—	—	—	—
240	32 (20)	117 (74)	—	2 (1)	7 (5)	67	14 (16)	60 (70)	13 (14)	70	50
229	26 (17)	120 (78)	—	3 (2)	8 (5)	76	14 (15)	68 (75)	9 (10)	76	56
220	36 (22)	105 (66)	—	3 (1)	19 (11)	74	11 (14)	76 (73)	10 (13)	92	71
Mean											
224	30 (20)	107 (72)	—	3 (1)	11 (7)	69	15 (17)	64 (71)	10 (12)	84	65

evenly distributed between the small myelin (A) and mitochondrial (C) fraction. Two experiments (a and b of Table 5), in which nitrogen determinations were also carried out showed that the nerve-ending fraction and the fraction P₂ from which it is derived also have the highest concentrations of ATP/mg. of nitrogen, and indeed, are the only fractions with a higher concentration than the original homogenate.

Use of more complex gradients. Interpolation of a layer of 1.0M-sucrose between the 0.8 and 1.2M layers split the B fraction into two fractions, B₁ and B₂, containing 33 and 66% of the ATP of the original fraction B respectively, but no further concentration of ATP relative to nitrogen was achieved by this procedure. In the electron microscope B₁ was seen to contain a higher proportion of microsomal particles, broken endings and endings of smaller size than B₂.

Subcellular distribution in white and grey matter. The results in Table 3 indicated that there was no marked difference between the 'stable' ATP content of grey and white matter. As the white matter does not contain nerve endings we were interested in the subcellular distribution of ATP in this tissue.

Table 5. Concentrations of adenosine triphosphate in subcellular fractions of guinea-pig brain

(a) and (b) refer to two separate experiments. For identification of symbols see Scheme 1.

Tissue fraction	Concn. of ATP (μg./mg. of N)	
	(a)	(b)
H	16.4	13.2
P ₁	10.8	9.0
P ₂	23.4	16.4
S ₂	—	6.8
P ₃	1.9	—
S ₃	2.7	—
A	7.8	7.9
B	29	20.3
C	11.9	11.1

Accordingly, samples of grey and white matter (cerebral cortex and internal capsule) weighing about 3 g. from two dogs were homogenized separately in 0.32M-sucrose and fractionated according to the usual procedure.

Table 6 gives the distribution of ATP and nitrogen in the various fractions as a percentage of the total ATP and nitrogen recovered in these fractions. The distribution of ATP and nitrogen in grey matter corresponds closely to their distribution in the guinea-pig fractions with a relative and absolute concentration in the nerve-ending fraction.

In the white matter the distributions are very different. Most of the nitrogen and ATP were recovered in fractions P₁ and A, whereas the nerve-ending fraction is, as expected, very small. From grey matter only fractions B and C showed a concentration of ATP higher than that of the original homogenate; fractions P₁ and A of white matter also showed a relative concentration of ATP. Not too much significance should be attached to the figures for the concentrations in fractions B and C as these contained only small amounts of ATP and nitrogen. Density-gradient separation and electron-microscope examination of fraction P₁ from white matter showed that it consisted largely of myelin fragments. There seems no doubt therefore that ATP is associated with the myelin fraction as well as with nerve endings, and the ATP found in fractions P₁ and A from whole brain is not accounted for simply by contamination with the nerve-ending fraction.

A further indication of the heterogeneous nature of 'stable' ATP is the presence of some ATP in the high-speed supernatant fraction (S₃). In experiments in which particle-free supernatants were prepared directly from sucrose homogenates by centrifuging the latter at 100 000 g for 3 hr., as much as 11% of the ATP of the original homogenate was recovered in the particle-free supernatant. This ATP must be bound to soluble protein, because it

Table 6. Subcellular distribution of 'stable' adenosine triphosphate in subcellular fractions of the grey and white matter of dog brain

The ATP and N content of each fraction is expressed as the percentage of the total recovered ATP or N found in that fraction. For identification of symbols see Scheme 1.

Fraction	Grey matter (cerebral cortex)			White matter (internal capsule)		
	ATP (%)	N (%)	% ATP:% N	ATP (%)	N (%)	% ATP:% N
P ₁	20	35	0.57	46	44	1.05
A	11	15	0.73	38	33	1.15
B	50	22	2.27	4	1	4*
C	8	7	1.14	2	1	2*
P ₃	1	8	0.12	4	6	0.67
S ₃	10	23	0.43	7	15	0.46

* Unreliable? (see comment in text).

Table 7. *Recovery of added adenosine triphosphate from sucrose homogenates of guinea-pig brain*

Treatment of homogenate before addition of ATP (24 µg./ml.)	Total ATP recovered (µg./ml.)		Added ATP recovered (as % of added ATP)
	No ATP added	ATP added	
(a) None	12.5	15	10
(b) Heated to 100° for 10 min.	4.5	23	77
(c) Made 10 % (w/v) with trichloroacetic acid	12.5	36	98

Table 8. *Effect of disruptive treatments on the content of adenosine triphosphate homogenates of guinea-pig brain*

Treatment	Loss of 'stable' ATP of homogenate (%)
Heating to 100° for 10 min.	60
Standing at 22° for 1 hr.	40
Standing at 4° for 7 hr.	25
Resuspension of loosely packed pellet	20
Resuspension of tightly packed pellet	60
Addition of Lissapol (0.01 %, w/v)	90

could not be detected unless first released by trichloroacetic acid and also because free ATP, as described in the next section, is rapidly destroyed in brain homogenates.

Nature of the 'stable' fraction of brain ATP

To obtain more information about the nature of the 'stable' fraction of brain ATP its stability was compared with that of added ATP. ATP was added at 0° to (a) untreated samples of sucrose homogenates of brain tissue and to samples that had been either (b) heated to 100° for 10 min. and then chilled to 0° or (c) made 10 % (w/v) with respect to trichloroacetic acid. A control series to which no ATP was added were also prepared. After 3 min. trichloroacetic acid in a final concentration of 10 % (w/v) was added to samples (a) and (b) and all samples were centrifuged to give supernatants in which ATP was estimated in the usual way. The results are given in Table 7. It will be seen that added ATP was rapidly destroyed at 0° in the presence of untreated homogenate (line a), but was almost fully recovered if trichloroacetic acid had been added first (line c). The fact that the recovery was also increased by heating the sample at 100° before addition of ATP (line b) suggests that the free ATP is destroyed by enzyme action. It follows that 'stable' ATP, including the fraction found in S₃, must be in some way protected against adenosine triphosphatase also present in the homogenate.

No systematic studies have been made of the release of ATP from the particulate fractions, but experience has shown that any form of disruptive treatment, however mild, results in a loss of ATP from the fraction (e.g. Table 8).

Effect of reserpine on the 'stable' ATP content of guinea-pig brain

In view of the well-known ability of reserpine to deplete the brain of pressor amines and adrenal-medullary and duodenal storage granules of both ATP and amines (Schümann, 1958; Prusoff, 1961), we were interested in the effect of reserpine on the amounts of 'stable' ATP of guinea-pig brain.

Accordingly three guinea-pigs were injected intravenously with reserpine (5 mg./kg.); 15 hr. later they were killed and the ATP and 5-hydroxy-tryptamine concentrations in the homogenate of whole brain and in the different fractions were determined. In all three animals the 5-hydroxy-tryptamine concentration in the initial homogenate was found to be decreased by 80–90 %. The ATP values found in the homogenate and in the different fractions are given in Table 9. The mean concentrations of fractions of nine normal guinea-pig brains and the concentrations found in the homogenate and primary fractions of the brain of a guinea pig that had been given an injection of pentobarbitone sodium intraperitoneally (2 mg./kg.) 2 hr. before decapitation are given for comparison.

The effect of reserpine injection on the 'stable' ATP fraction can be summarized as follows. The ATP concentration in the initial homogenate was higher than normal. This seems to be due to a sedative effect of the drug, as the same effect was obtained with pentobarbitone in one guinea pig and two dogs. The recoveries in the primary fractions are lower than in normal guinea pigs but the amount of ATP/g. wet wt. in each of the fractions was nearly the same as in normal guinea pigs. This would be explained if the additional ATP in the homogenates from treated animals were not of the 'stable' type.

The recovery and distribution pattern in the subfractions showed no striking difference from normal although there was some indication of a slight increase in the mitochondrial ATP.

DISCUSSION

Our results do not permit any conclusion to be drawn about a specific role of ATP in the storage of pressor amines.

Table 9. *Effect of reserpine and pentobarbitone on concentrations of adenosine triphosphate in subcellular fractions of guinea-pig brain*

The concentration of ATP in each fraction (symbols as in Scheme 1) is given as $\mu\text{g./vol.}$ of fraction equivalent to 1 g. of tissue and (in parentheses) as a percentage of the total recovered ATP in the fraction. Normal values are from Table 4 and are the averages of five experiments.

H	P ₁	P ₂	S ₂	A	B	C	Recovery of ATP (%)	
							In primary fractions	In sub-fractions
Normal animals								
205	29 (16)	101 (63)	34 (21)	15 (17)	64 (71)	10 (12)	81	84
Reserpine-treated animals								
420*	46 (17)	172 (67)	45 (16)	20 (13)	93 (63)	37 (24)	62	90
354	18 (12)	112 (73)	23 (15)	14 (15)	59 (63)	19 (22)	48	76
320	21 (14)	94 (61)	33 (22)	—	—	—	46	—
Pentobarbitone-treated animals								
340	21 (15)	90 (66)	25 (19)	—	—	—	42	—

* Young animal, wt. 200 g.

The regional distribution of 'stable' ATP shows no correlation with that of hydroxytryptamine or noradrenaline. Whereas 'stable' ATP is distributed fairly uniformly in both grey and white matter, both hydroxytryptamine and noradrenaline show marked regional variations in distribution, being present in highest concentration in the hypothalamus and in lower concentrations in the medulla, caudate nucleus, thalamus, cerebral cortex and cerebellum (Bertler & Rosengren, 1959; Paton, 1958).

Reserpine failed to deplete the brain of 'stable' ATP in doses that almost completely depleted it of hydroxytryptamine. This is in marked contrast with the effect of reserpine on the adrenal medulla (Schümann, 1958) and duodenal mucosa (Prusoff, 1961).

In the nerve endings ATP was found to be present in much higher concentration (about 210 $\mu\text{m-moles/g.}$ of tissue) than hydroxytryptamine (about 1 $\mu\text{m-mole/g.}$ of tissue); thus the ratio of ATP to amine was about 200:1 and greatly exceeded the 1:4 ratio to pressor amines found in non-nervous storage granules. Thus if there is a fraction of ATP concerned with the storage of pressor amines in stoichiometric amounts similar to those found in non-nervous storage granules, this fraction would be completely masked by another fraction of ATP that is evidently not concerned directly with such storage. This is somewhat analogous to findings with platelets, which are also believed to contain a reserpine-insensitive fraction of ATP which raises the ATP to amine ratio well above 0.25 (Born, Ingram & Stacey, 1958).

The nature and role of the 'stable' ATP is obscure. It is clearly heterogeneous in origin. Part of it may consist of ATP bound to membranes, and be concerned with protein synthesis or ionic movements, which are now believed by many investigators to be coupled to ATP breakdown. The

soluble bound ATP of the supernatant fraction might represent ATP bound to soluble ATP-requiring enzymes. The high concentration in the nerve endings might have several alternative explanations.

First, ATP might be involved in the synthesis or binding of transmitter substances other than the pressor amines, among them acetylcholine. However, the regional distribution of acetylcholine, either alone or summed with that of hydroxytryptamine and noradrenaline, does not correlate with that of ATP. Secondly, ATP may itself be a transmitter substance, as suggested by Holton (1959). Thirdly, the high concentration of ATP may simply reflect the survival in this fraction of the organized cytoplasm of a region of the neurone which would be expected to have a high rate of metabolism. Further work will be required before the significance of the ATP of the nerve-ending fraction is fully understood.

SUMMARY

1. The contents of adenosine triphosphate of homogenates of normal guinea-pig brain in 0.32M-sucrose and of fractions derived therefrom have been determined by the firefly method and compared with those of animals killed by immersion in liquid nitrogen and of animals pretreated with drugs.

2. After a rapid initial post-mortem fall, brain adenosine triphosphate reached a relatively stable value that was unaffected by homogenization. About half of this 'stable' adenosine triphosphate was recovered in a fraction consisting mainly of pinched-off nerve endings, the remainder in myelin, mitochondria and the soluble supernatant.

3. The amount of adenosine triphosphate in the nerve-ending fraction was found to be greatly in excess of the 1:4 ratio of adenosine triphosphate to pressor amine expected if the function of adenosine

triphosphate there was to bind amines, as it is believed to be in other amine-storage particles. The lack of effect on the content of adenosine triphosphate of doses of reserpine which effectively depleted the brain of 5-hydroxytryptamine and the lack of correlation in the regional distributions of adenosine triphosphate and pressor amines are further reasons for thinking that the 'stable' fraction of adenosine triphosphate studied is not mainly concerned with amine storage.

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The Formation of 2,6-Dichlorobenzonitrile from Related Compounds in Plants

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The discovery of the herbicidal properties of 2,6-dichlorobenzonitrile (Barnsley & Rosher, 1961; Koopman & Daams, 1960) led to the synthesis of a number of related compounds, containing a 2,6-dihalogenated phenyl nucleus, which had the same highly specific toxicity symptoms (Milborrow, 1963) as 2,6-dichlorobenzonitrile when applied to plants. The similarity of the plant response obtained with 2,6-dichlorobenzonitrile, 2,6-dichlorobenzaldoxime and α -amino-2,6-dichlorobenzaldoxime suggested that the last two compounds were converted into 2,6-dichlorobenzonitrile. A similar reaction has been described by Ahmad & Spenser (1960), who suggested that the indolylacetonitrile precursor obtained by Housley & Bentley (1956) was the oxime of indolylpyruvic acid as synthetic indolylpyruvic acid oxime was readily converted into indolylacetonitrile under mild chemical conditions. The identity of the metabolite produced in cultures of *Pseudomonas putrefaciens* and field soil, to which the 2,6-dichlorobenzaldoximes had been added, has therefore been investigated.

MATERIALS AND METHODS

Chemicals. 2,6-Dichlorobenzonitrile was purified (m.p. 145°) by repeated recrystallization from acetone. The 2,6-dichlorobenzaldoximes were obtained from 'Shell' Research Ltd.

Analytical methods. The solutions were analysed for 2,6-dichlorobenzonitrile by gas-liquid chromatography (Goodwin, Goulden & Reynolds, 1961) but with a 122 cm. column of 5% 'Epikote' resin 1001 (Shell Chemical Co. Ltd.) on 100-120 mesh Celite (J. Jay's, Ewell, Surrey) at 163°, and also by reversed-phase paper chromatography with phenoxyethanol as stationary phase and iso-octane as the mobile one. The compounds were detected by exposure to ultraviolet light after spraying with 5% (w/v) silver nitrate in phenoxyethanol (Mitchell, 1958). Nitrogen was determined by a Kjeldahl method (Knowles & Watkin, 1950).

Soil-incubation experiments. α -Amino-2,6-dichlorobenzaldoxime solutions (1 ml.) were added to boiling tubes containing 20 g. of a brick-earth field soil and incubated at 25°. After appropriate intervals the contents of each tube were mixed with 50 g. of anhydrous sodium sulphate to form a dry powder which was extracted with 25 ml. of