Observations on Brain Phosphatases

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The presence of an 'ordinary' phosphatase (i.e. phosphomonoesterase) in brain was reported by Vondracek (1927). Activities, however, were four to fifteen times lower than in the kidney (King, 1931; Macfarlane, Patterson & Robison, 1934). Giri & Datta (1936) showed that acetone-dried powders prepared from brain can hydrolyse both β -glycerophosphate and hexose diphosphate at the optimum pH values of 5.0 and 9.4. Brain tissue also hydrolyses adenosine 5'-phosphate (Reis, 1937), adenosine triphosphate (ATP) (Dubois & Potter, 1943), and inorganic pyrophosphate (Gordon, 1950). Distribution of phosphatases in different regions of ox and human brain has been studied chemically by Fleischhacker & Yates (1936) and Fleischhacker (1938), and certain variations in activity were noted. Other investigations of phosphatase activity in the brain and nervous system have been carried out by the use of histological techniques (Landow, Kabat & Newman, 1942; Wolf, Kabat & Newman, 1943; Naidoo & Pratt, 1951, 1952).

The present investigation was undertaken to examine more extensively the distribution of phosphomonoesterase and pyrophosphatase (PPase) in the brain, and to obtain values for normal human brains for comparison with brains obtained *post mortem* from cases of psychosis. Some evidence has been presented by Binkley & Olson (1950) that inorganic pyrophosphate and ATP may be acted upon by the same enzyme system in brain, and since this point was of possible importance in interpreting the results of PPase measurements, experiments were carried out on rat-brain preparations to compare the properties of adenosine triphosphatase (ATPase) and PPase. An account of this work is included in the present paper. These experiments support the conclusion reached by Gore (1952), using guinea pig brain, that ATPase and PPase are separate systems.

METHODS

Enzyme preparations. Aqueous dispersions of brain, and suspensions made from acetone-dried powders of brain, were used as sources of enzyme.

Acetone-dried powders were prepared from whole rat brains. The brains were ground with ice-cold acetone (2-3 ml.) in a Potter-Elvehjem (1936) homogenizer. The solid matter was then washed successively with ice-cold acetone, aqueous acetone, and ether, and finally dried,

according to the procedure previously employed (Gordon, 1950). When required for use, the dry powder (50-100 mg.) was ground with glycerol (2.5-5.0 ml.) to a paste and then made into a suspension in water.

Aqueous dispersions of rat-brain tissue were made by grinding whole rat brains with ice-cold 0.9% NaCl (2-3 ml.). For phosphomonoesterase measurement the dispersion was diluted to 150 mg./ml. wet weight and dialysed in a Visking cellophane tube ($\frac{1}{4}$ in. diameter) against running water for 16 hr., to remove inorganic phosphate. For PPase and ATPase measurements the dispersions were diluted to 10-15 mg./ml., but were not dialysed.

Normal human brains were obtained from the Bristol General Hospital, from cases where a post-mortem examination was conducted within 24 hr. of death. Such brains were packed in cooled porcelain containers, sealed over with cellophane, transported to the laboratory and dissected as soon as possible afterwards. Small quantities (up to 1 g. where possible) of tissue from various regions were dissected out and transferred to tared stoppered weighing bottles, which were then re-weighed. The tissue was transferred with the minimum amount of 0.9% NaCl to suitable tubes, ground as usual, and made to the appropriate concentrations; the dispersions which were required for phosphomonoesterase measurement were dialysed, as above.

Sheep brains, obtained from the abattoir, were packed on ice, transported to the laboratory and dissected and ground in the same manner as human brain.

Enzyme assay. Estimations of phosphomonoesterase and PPase were carried out at 37° in the same general manner as previously described (Gordon, 1950) with certain variations.

PPase activity was measured at pH 7.4 in veronal buffer medium. Tissue dispersions were used at a final concentration of 2.5 mg./ml. wet weight, a Mg^{2+} concentration of 0.008 M and a substrate concentration of 0.001 M. Acetone-powder preparations were used as before (Gordon, 1950), but with a substrate concentration of 0.001 M. In the experiments on sheep and human brain, cysteine (0.01 M) was included as an activator.

For phosphomonoesterase measurements the tissue dispersions were used at a final concentration of 18 mg./ml. wet weight. Sodium β -glycerophosphate (0.0075 M) was used as substrate. Acid phosphatase was measured in presence of acetate buffer (pH 5.0) using a 2 hr. incubation period. Alkaline phosphatase was measured in presence of veronal buffer (pH 9.4) using a 1 hr. incubation period.

ATPase activity was estimated at pH 7.4, using veronal buffer. Brain dispersions were used at a final concentration of 2.5-5.0 mg./ml. wet weight. The ATP concentration was 0.003 M, and Mg^{2+} (0.002 M) was also present. An incubation period of 15 min. was allowed. Acetone-dried powder preparations were used at a final concentration of 2.5-5.0 mg./ml., with a Mg^{2+} concentration of 0.01 M.

Table 1. *Effects of magnesium and calcium ions on adenosine triphosphatase and pyrophosphatase of rat brain*

Ion	Concentration (M)	Activity (mm substrate/30 min./100 g.)		
		PPase (dispersion)	ATPase (dispersion)	ATPase (acetone prep.)
None		0	10.8	0
Ca ²⁺	1.5 × 10 ⁻⁴	—	20.5	0
Ca ²⁺	10 ⁻³	0	22.0	0
Mg ²⁺	3 × 10 ⁻⁴	—	28.3	—
Mg ²⁺	2 × 10 ⁻³	23.5	46.2	—
Mg ²⁺	4 × 10 ⁻³	32.0	46.0	3.8
Mg ²⁺	8 × 10 ⁻³	34.5	—	—
Mg ²⁺	10 ⁻²	—	—	4.5
Mg ²⁺	1.6 × 10 ⁻²	34.5	—	—

All results are expressed as mm substrate/30 min./100 g. wet-weight tissue. It was confirmed by independent experiments that the progress of the phosphomonoesterase reaction at pH 5.0 and 9.4 was linear over the limits of the assay times employed. In the case of ATPase it was assumed that only one orthophosphate group was liberated during the short period of incubation employed.

ATP (barium salt), obtained from Boots Pure Drug Co., was washed with acetate buffer (pH 4.0, 0.1M) as recommended by Kornberg (1950), and converted into the sodium salt as described by Bailey (1942). The concentration was checked by a 7 min. hydrolysis test.

RESULTS

Adenosine triphosphatase activity

The present findings for rat brain agree broadly with those of Gore (1952) for guinea-pig brain. Activity was maximum at pH 7.4 over a concentration range of 3–5 × 10⁻³M. Hydrolysis was linear up to 23 % decomposition. It has previously been observed (Feldberg & Mann, 1945; Meyerhof & Wilson, 1948; Gore, 1952) that ATPase activity of brain tissue is destroyed by acetone drying. In the present study, acetone powders showed much lower activity than tissue dispersions (approximately 8–16 %, referred to weight of original tissue). The activity was still measurable, however, and a number of experiments recorded later in the paper were performed on suspensions of acetone-dried powders.

Effects of magnesium and calcium ions on pyrophosphatase and adenosine triphosphatase

Brain PPase, in common with the PPases from various other sources, is activated by Mg²⁺ and inhibited by Ca²⁺ (Gordon, 1950). ATPase in brain has previously been reported to be activated by Ca²⁺ (Dubois & Potter, 1943; Feldberg & Mann, 1945; Epelbaum, Sheves & Kobylin, 1949), but Gore (1952) was unable to demonstrate this. It can be seen from Table 1 that dispersions of fresh brain showed appreciable ATPase activity even in absence of an activating ion; presence of Ca²⁺ increased this activity by more than 100 %, but

Table 2. *Effects of dialysis and cysteine on pyrophosphatase and adenosine triphosphatase activities of rat brain*

	% change	
	PPase	ATPase
Activity lost on storage at 0°	15.5	25.7
Activity lost on dialysis	87.2	51.3
Activation of fresh enzyme by 0.01M cysteine	16.5	21.7
Activity restored to dialysed enzyme by 0.01M cysteine	42.0	55.5

a much greater effect was shown by Mg²⁺ at an optimum concentration of 0.002M. Acetone-powder preparations showed some ATPase activity, at a greatly reduced level, only in presence of Mg²⁺ ions; Ca²⁺ ions were ineffective. In contrast, PPase in fresh tissue dispersions was not activated by Ca²⁺ but was activated best by Mg²⁺ at 0.008M, compared with 0.015M previously found with acetone preparations (Gordon, 1950). Gore (1952) found that ATPase in guinea pig brain dispersions was inhibited by Ca²⁺ at 6 × 10⁻⁴M. This difference, however, is probably accounted for by the different conditions employed. Gore's assays were carried out in presence of glycylglycine buffer, and it was noted that under these conditions the ATPase activity may be three times as high as with veronal buffer (Dubois & Potter (1943), who demonstrated an activation by Ca²⁺, used veronal buffer).

Effects of dialysis

When brain dispersions (10 mg./ml.) were dialysed against a large volume of water at 0° for 18 hr., considerable loss of PPase activity occurred, while storage of the preparation at 0° for the same time resulted in relatively only slight loss (Table 2). This lability on dialysis does not appear to be due to loss of a diffusible coenzyme, since attempts to demonstrate reactivating properties in the dialysate have failed. It is of interest, however, that cysteine, which causes moderate activation of the undialysed enzyme, also restores a large proportion of the lost activity. ATPase on dialysis also showed loss of

activity, which however was much less marked; in this case, only slight restoration of activity was brought about by cysteine, which also caused slight activation of the undialysed enzyme.

Fractionation of pyrophosphatase and adenosine triphosphatase

Experiments were attempted to determine whether a partial separation of ATPase and PPase could be achieved. 10 ml. portions of a fresh rat-brain dispersion (100 mg./ml.) were first treated with saturated ammonium sulphate solution to bring the mixture to a definite saturation level. After standing for 30 min. at 0°, the precipitates were centrifuged and resuspended in water (8 ml.) and, after diluting suitably, the activities were determined as usual. Control assays on the original

dispersion were also carried out. It was confirmed by experiment that ammonium sulphate at 10 mg./ml. concentration did not directly affect the activities. Results shown in Table 3 indicate that PPase and ATPase both tend to concentrate mainly in the 30–40% saturation fractions, but whereas only 20% of the PPase activity is found in the 40% fraction, ATPase is found to the extent of 40% in the 20% fraction and 64% in the 30% fraction. A difference between ATPase and PPase was also seen when ethanol fractionation was tried. Though maximum concentration was found in both cases at 40% saturation, 38% of the PPase and only 21% of the ATPase activity were found in this fraction.

Effects of inhibitors

Gore (1952) found that ATPase and PPase of guinea pig brain differed in their behaviour towards certain inhibitors. In the present study, experiments were carried out on rat brain, using aqueous dispersions and acetone-dried preparations, to determine whether there are differences in sensitivity between the two enzymes towards various compounds. The results reported in Table 4 would appear to give some indication that such differences may exist. Zinc, iron or copper sulphate, mercuric chloride, sodium fluoride, sodium cyanide and mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide) all brought about strong inhibition of PPase activity in the aqueous dispersions, at concentrations which caused only slight or moderate inhibition of ATPase. The differences were less marked in some instances (especially zinc sulphate and sodium fluoride) when acetone preparations were used for the activity measurements. Alloxan and

Table 3. *Fractionation of pyrophosphatase and adenosine triphosphatase activities of rat brain by ammonium sulphate or ethanol*

(10 ml. of 100 mg./ml. dispersion treated with different concentrations of $(\text{NH}_4)_2\text{SO}_4$ or ethanol and centrifuged; precipitates suspended in 8 ml. water.)

Reagent	Concentration (%, w/v)	% Activity found in precipitate	
		PPase	ATPase
$(\text{NH}_4)_2\text{SO}_4$	10	8.4	21.0
$(\text{NH}_4)_2\text{SO}_4$	20	9.8	40.0
$(\text{NH}_4)_2\text{SO}_4$	30	12.6	64.0
$(\text{NH}_4)_2\text{SO}_4$	40	20.2	71.0
Ethanol	20	1.7	3.3
Ethanol	40	38.2	21.1
Ethanol	60	38.2	23.3
Ethanol	80	41.2	23.3

Table 4. *Inhibition of rat brain pyrophosphatase and adenosine triphosphatase by various compounds*

Inhibitor	Concentration (M)	Inhibition (%)			
		Dispersion		Acetone preparation	
		PPase	ATPase	PPase	ATPase
ZnSO ₄	4×10^{-5}	98	0	97	45
ZnSO ₄	4×10^{-6}	82	0	35	8
FeSO ₄	4×10^{-4}	96	28	100	43
FeSO ₄	4×10^{-5}	93	21	87	41
CuSO ₄	5×10^{-5}	100	51	100	52
CuSO ₄	2×10^{-5}	93	0	100	17
HgCl ₂	2×10^{-5}	97	54	97	52
HgCl ₂	2.5×10^{-6}	91	6	23	0
CaCl ₂	1.5×10^{-3}	99	10	87	79
CaCl ₂	2.5×10^{-4}	75	16	66	54
NaF	1×10^{-4}	85	6	93	47
NaF	5×10^{-5}	71	0	76	16
NaCN	2×10^{-2}	31	0	43	0
Mapharside	2×10^{-5}	78	22	77	0
Mapharside	1×10^{-6}	25	6	30	0
Alloxan	5×10^{-3}	99	48	96	63
Alloxan	5×10^{-4}	71	23	80	44
Formaldehyde	2.5×10^{-2}	87	47	94	89
Formaldehyde	2.5×10^{-4}	23	0	36	16
Suramin	4×10^{-4}	84	62	75	54

formaldehyde inhibited PPase more strongly than ATPase, but the differences were not as great as with most of the other substances. Suramin inhibited both enzymes to a comparable extent. Calcium chloride inhibited PPase; it did not greatly inhibit ATPase in the aqueous dispersions, but caused marked inhibition of ATPase in the acetone-powder preparations. A strict comparison between the behaviour of the dispersions and acetone preparations is not really possible, since both preparations contained much inert material, and a large proportion of the ATPase in the acetone preparations would most likely have been destroyed by the acetone treatment. It is of interest, however, that Gore (1952) also found that sodium fluoride, copper sulphate and alloxan exerted a relatively greater inhibitory effect on PPase than on ATPase.

Distribution of phosphatases in brain

Tables 5 and 6 show representative results for the phosphomonoesterase and PPase levels in different regions of sheep and human brain. It has not been possible, so far, to examine a sufficient number of human brains to be able to deduce normal levels for

comparison with abnormal brains, as there were, in some cases, rather large differences between individual brains. Relative variations throughout the same brain, however, showed a fairly consistent pattern, and the general trend can be seen in the examples quoted.

In sheep brain, the highest values for phosphomonoesterase were found in the occipital cortex, and for PPase in the superior colliculus. The corpus callosum, a conducting tissue, showed much lower activities than the other regions for acid and alkaline phosphatase, but not for PPase. Values for the medulla were in no case significantly lower than in the nucleated regions. These results do not offer evidence of a simple relation between enzyme activity and structure or function.

With human brains the pattern was different; some interesting variations in activity of PPase may be noted. High values occurred in the caudate nuclei, the thalamus, the globus pallidus, the frontal cortex, the putamen, the hypothalamus and the floor of the fourth ventricle, all of which are nucleated regions of high functional significance. Much lower values were found in the medulla (olive)

Table 5. *Distribution of phosphatases in sheep brain*

(Activities expressed as mM substrate/30 min./100 g. tissue.)

Region	Alkaline phosphatase		Acid phosphatase		PPase	
	Brain 1	Brain 2	Brain 1	Brain 2	Brain 1	Brain 2
Caudate nuclei	2.30	1.93	0.36	0.73	28.6	29.6
Superior colliculus	2.45	2.21	0.39	0.54	41.6	43.2
Medulla	1.94	2.46	0.26	0.49	31.1	28.7
Thalamus	1.94	2.32	0.28	0.39	29.1	29.1
Occipital cortex	2.95	2.84	0.44	0.81	32.2	34.1
Corpus callosum	0.61	1.03	0.16	0.07	34.2	29.3
Hypothalamus	2.19	1.98	0.29	0.49	38.6	41.0

Table 6. *Distribution of phosphatases in normal human brain*

(Activities expressed as mM substrate/30 min./100 g. tissue.)

Region	Alkaline phosphatase				Acid phosphatase				PPase			
	Brain 1	Brain 2	Brain 3	Brain 4	Brain 1	Brain 2	Brain 3	Brain 4	Brain 1	Brain 2	Brain 3	Brain 4
Caudate nuclei	1.65	0.82	—	—	0.52	0.72	—	—	26.5	20.6	—	—
Medulla (pyramids)	1.05	0.69	—	—	0.42	0.25	—	—	13.8	11.5	—	—
Medulla (olive)	1.19	0.64	—	—	0.34	0.41	—	—	17.8	18.1	—	—
Thalamus	1.77	0.82	—	—	0.55	0.75	—	—	30.1	30.0	—	—
Globus pallidus	1.34	1.15	—	—	0.41	0.67	—	—	28.1	26.6	—	—
Frontal cortex	2.06	1.34	—	—	0.55	0.72	—	—	28.3	29.4	—	—
Prefrontal cortex	—	—	1.77	1.46	—	—	0.67	0.66	—	—	21.3	20.1
Motor cortex	—	—	1.34	1.17	—	—	0.64	0.61	—	—	23.0	17.3
Sensory cortex	—	—	1.50	1.50	—	—	0.71	0.62	—	—	27.0	20.6
Temporal cortex	—	—	1.77	1.80	—	—	0.68	0.69	—	—	22.9	20.0
Palaeo-cerebellar cortex	—	—	1.45	1.20	—	—	0.86	0.73	—	—	36.5	48.6
Neocerebellar cortex	—	—	1.51	1.24	—	—	0.87	0.69	—	—	42.0	50.7
Hippocampus	—	—	1.32	1.22	—	—	0.78	0.63	—	—	23.9	23.7
Corpus callosum	1.05	0.43	—	—	0.26	0.37	—	—	17.3	16.0	—	—
Putamen	1.96	1.17	—	—	0.57	0.67	—	—	33.5	28.1	—	—
Hypothalamus	1.72	0.88	—	—	0.58	0.80	—	—	32.3	29.4	—	—
Fornix	0.60	0.54	—	—	0.28	0.27	—	—	14.5	13.4	—	—
Floor of 4th ventricle	1.58	0.60	—	—	0.55	0.44	—	—	29.0	24.2	—	—

region, a nucleated region with much conducting tissue, and in the corpus callosum, a definite conducting region. Lowest values were found in the medulla (pyramid) and the fornix, which are also conducting regions. There were no marked or significant differences between the different areas of cerebral cortex, but it is of interest that values in the cerebellar cortex were markedly higher than in the cerebral cortex.

Alkaline phosphatase of human brain also appears to be lowest in the conducting regions. There was appreciable variation between the nucleated regions, however; the floor of the fourth ventricle (brain 2, Table 5) was not much more active than the fornix. The caudate nuclei, the thalamus and the hypothalamus gave intermediate values, while the highest values were given by the globus pallidus, the prefrontal cortex and the putamen.

Acid phosphatase also showed lowest values in the conducting regions, but did not show some variation between nucleated areas as alkaline phosphatase did.

DISCUSSION

It will be noted that, though there are certain similarities between the properties of ATPase and PPase (notably in regard to their activation by cysteine), in other respects there are differences. The partial separation of the two activities achieved by precipitation with ammonium sulphate or ethanol is in agreement with Gore's (1952) success in separating the activities by extraction procedures. Some caution is necessary in drawing conclusions from the inhibition experiments, since crude enzyme preparations only were used, and the results may have been influenced by inert material (especially protein). There appears to be some likelihood that PPase is more readily inhibited than ATPase by most of the compounds tested, though it would be necessary to study this problem using purified enzyme preparations, before any real conclusions could be drawn from such differences. The observations reported, however, tend to support Gore's (1952) conclusions regarding the non-identity of the PPase and ATPase systems in brain.

The importance of enzyme distribution studies in brain has been emphasized by Ashby (1951) who reported wide variations in carbonic anhydrase activity between different regions. In the present study, the PPase levels show a distribution pattern which is related to the histological features of the different regions. Highly nucleated regions in

general are much more active than the conducting regions, and this factor appears to account mainly for the relative differences observed. At the same time, the PPase values for cerebellar cortex are considerably higher than for cerebral cortex; it does not seem likely that this phenomenon could be accounted for by the small histological difference between these types of tissue. The possible significance of the PPase distribution pattern is not apparent at present, since little is known about the metabolic role of PPase.

The same tendency for higher values to occur in nucleated regions, and lower values in conducting regions, is seen in the distribution of acid and alkaline phosphatase activity. It is of interest in this connexion that Richter & Hullin (1951) found a strong alkaline phosphatase reaction in cell nuclei isolated from cerebral cortex. Naidoo & Pratt (1951) demonstrated histologically that acid phosphatase activity, with glycerophosphate as substrate, was localized mainly in the neuronal cytoplasm; the cerebral cortex showed cells clearly defined by the staining. It seems probable on the whole that a better idea of the role of the phosphomonoesterase in brain will be obtained by studying the variations between different cellular layers, rather than between whole regions; some interesting observations of this type have already been made by Scharrer & Sinden (1949).

SUMMARY

1. The pyrophosphatase and adenosine triphosphatase activities of rat-brain dispersions may be partially separated by fractionation with ammonium sulphate or ethanol. Both enzymes lose activity on dialysis, and both are activated by cysteine.
2. Pyrophosphatase activity in crude preparations obtained from rat brains is strongly inhibited by a number of substances (metal salts, mapharside, formaldehyde and suramin), most of which exert relatively little effect on adenosine triphosphatase activity.
3. The distribution of phosphomonoesterase and pyrophosphatase activities in different regions of human and sheep brain has been studied. Activities tend to be high in the nucleated regions, and low in conducting regions.

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Biosynthesis of Choline in the Seedling of the Chick-pea (*Cicer arietinum*)

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The problem of methyl-group biosynthesis in the plant has not received as much attention as in the animal. Amongst important papers on the subject are those showing the formation of the methyl groups of hordenine and choline from formate by sprouting barley (Kirkwood & Marion, 1951) and of the methyl carbon of nicotine from formate and the methyl group of methionine by *Nicotiana rustica* L. (Brown & Byerrum, 1952). Barrenscheen & Pany (1941) and Barrenscheen & Valyi-Nagy (1942) observed the methylation of guanidoacetic acid to give creatine by 7-day-old wheat seedlings and also found that added methionine increased creatine synthesis sixfold to eightfold. They observed creatine synthesis to be an aerobic process where addition of hydrogen acceptors like cystine could not replace oxygen. They also noted that the transfer of the methyl group of methionine was accompanied by oxidation of the sulphur. Steensholt (1946), however, found that etiolated wheat germs were unable to use methionine for the methylation of ethanolamine to choline.

This paper describes experiments showing how the biological elaboration of the important methyl compound, choline, by the germinating seedlings of *Cicer arietinum* is influenced by certain inorganic salts, vitamins and by some organic compounds already known to be concerned with the biochemistry of methyl groups elsewhere.

EXPERIMENTAL

Technique of germination. 16–20 g. of seeds of *C. arietinum* were soaked in distilled water or in an aqueous solution of the test substance (hereafter designated as 'germinating fluid') for 12 hr. and then allowed to germinate on a sand bed in large baking dishes at room temperature (25–28°). At the end of the germination period, the seedlings, after removal of the seed coat, were dried and choline was determined.

Determination of choline. An adaptation of the methods described by Luecke & Pearson (1944) and McIntire, Sweigert and Elvehjem (1944) was used. The finely minced seedlings (1 g.) were autoclaved with 10 ml. of 3N-HCl for 2 hr. at 15 lb./sq. in. The hydrolysate after neutralization with NaOH was passed through a column of Decalso at a rate of 6 to 10 drops/min. The adsorbed choline was then eluted with two 10 ml. portions of 5% NaCl. 5 ml. of 2% ammonium reineckate in methanol were added to the eluate, which was kept at -4° for 24 hr. to allow complete precipitation of choline reineckate. The precipitate was then collected on an asbestos pad and washed three times with 2 ml. portions of ice-cold ethanol. Air was drawn through the pad until the precipitate was dry. The precipitate was then washed down with acetone and the washings were made up to 25 ml. The colour due to choline reineckate was measured in a photoelectric (Klett-Summerson) colorimeter. A standard, with 4 mg. of choline chloride, and a reagent blank were run with every determination.

Each set of experiments described below was run five times, and every determination done in triplicate. No variation over 5% was noticed and the results presented are average values.