A STUDY OF THREE PHOSPHOMONOESTERASES IN THE DOG'S CEREBRUM

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

by

Nabil Wakid

Department of Biological Chemistry
American University of Beirut

1953

TABLE OF CONTENTS

.. ..

I N T R O D U C T I O N

The Occurrence of Phosphatases in the Brain

The interest in brain phosphatases began in the late nineteen thirties when Giri and Datta (1,2) reported two phosphomonoesterases in that organ which hydrolyzed beta-glycerophosphate at different pH optima: 9-9.6 and 5-5.4. Two years later Cedrangolo and Ruffo (3) succeeded in separating the two activities by fractionating with 50% ethanol, thus establishing their identity as two isodynamic monoesterases. Similar phosphomonoesterases, either together or alone had been previously reported in other animal organs like the liver, kidney (4), serum (5) and red blood cells (6).

At about the same time Reis (7,8) discovered another phosphomonoesterase in brain that was specific only for 5' phospho - nucleotides. Although the other non-specific phosphatases also split 5' nucleotides this specific monoesterase was recognized by the fact that it had a different pH optimum and that it hydrolyzed adenylic acid much more rapidly than beta-glycerophosphate.

The Intracellular Distribution of Phosphatases.

The distribution of these enzymes has already been studied on liver. The technique used has been that of differential centrifugation introduced by Benseley and Hoerr (9) and later improved by Claude (10), Schneider and Hogeboom (11) and others.

Using 0.88M sucrose as a suspending medium Novikoff et al (12) studied the intracellular distribution of the enzymes in liver acting on phenylphosphate at pH 4.5 and 9.1, on adenosine 5' phosphate (A5P) and on adenosine triphosphate (ATP).

Palade (13), also Berthet and de Duve (14) studied the distribution of the acid phosphatase acting on beta-glycerophosphate (optimum pH5) and found it to be associated with the mitochondrial fraction of the liver cell.

In the brain, however, only the nuclei of the cortex have been studied as to phosphatase content (15). In a study of respiratory enzymes Brody and Bain (16) separated the cytoplasm of brain cells by differential centrifugation into three fractions: mitochondria, microsomes, and soluble substance of the cell (the final supernatant). No such study has yet

been made on the distribution of the phosphatase of brain.

Differential Centrifugation of Cytoplasmic Particles. In the present study the centrifugal speeds for fractionating the cytoplasm were chosen more or less arbitrarily since, as shown by Chantrenne (17) on mouse liver, one can obtain from the cytoplasm a continuous spectrum of particles of varying size, the larger ones at the lower centrifugal speeds. The arbitrary selection was done on the assumption that this may also be the case with brain particles. The resulting fractions are named in terms that describe the conditions under which they are obtained rather than by such restricted terms as mitochondria and microsomes.

Preparation of Tissue & Differential Centrifugation. The animal (mature dog) was anesthetized with nembutal or ether. The skull was then carefully opened and the cerebrum removed, immediately chilled in a beaker of cracked ice and taken to the cold room (1-4°C) where it was freed as much as possible from its membranes and the blood vessels surrounding it.

The tissue was weighed, crushed in a mortar, and then homogenized with an equal volume of 0.25 M sucrose (Mallinckvodt) in an all-glass Potter-Elvehjem tube (18). More sucrose solution was added to the homogenate to make approximately a 1:3 (w/v) suspension of the original tissue. This was again passed through the homogenizer to insure maximum cell breakage. The resulting homogenate was strained through several layers of flannel gauze to remove any unbroken tissue. More sucrose solution was added to make a final suspension of 1:5 (w/v). The volume of the homogenate thus obtained was measured, an aliquot having been reserved for enzymatic assay and nitrogen determination.

The homogenate was centrifuged at 1200 times gravity (1200 G.) for 10 minutes the supernatant being carefully pipetted out without distur-

bing the residue. The residue obtained, G 1.2/10*, was resuspended by shaking in about 5 times its volume of 0.25 M sucrose and sedimented again with the same centrifugal speed for the same duration. The washings were added to the supernatant, this washing procedure being repeated once more.

The supernatant, S 1.2/10, was then centrifuged at 5000G for 15 minutes. The particles 1.2/10 G 5/15 were washed once as above. The supernatant was centrifuged at 20,000 G for 30 minutes, these particles being washed once. The supernatant, S 20/30 was centrifuged at 30,000 G for 90 minutes yielding a residue 20/30 G 30/90 and a final supernatant S 30/90.

Any scum on the surface was included with its supernatant. The residue 20/30 G 30/90 being compact was not washed by resuspension but merely by rinsing very gently with sucrose solution. The residue adhering to the bottom of the centrifuge tube was not disturbed.

The particles obtained were resuspended in ten volumes of 0.25 M sucrose. This suspension could be stored in the refrigerator for about two weeks without spoiling.

---

* This abbreviated method of naming the various fractions describes at the same time the conditions under which they were obtained. Thus 1.2/10 G 5/15 refers to the particles sedimented between a centrifugation at 1200 G

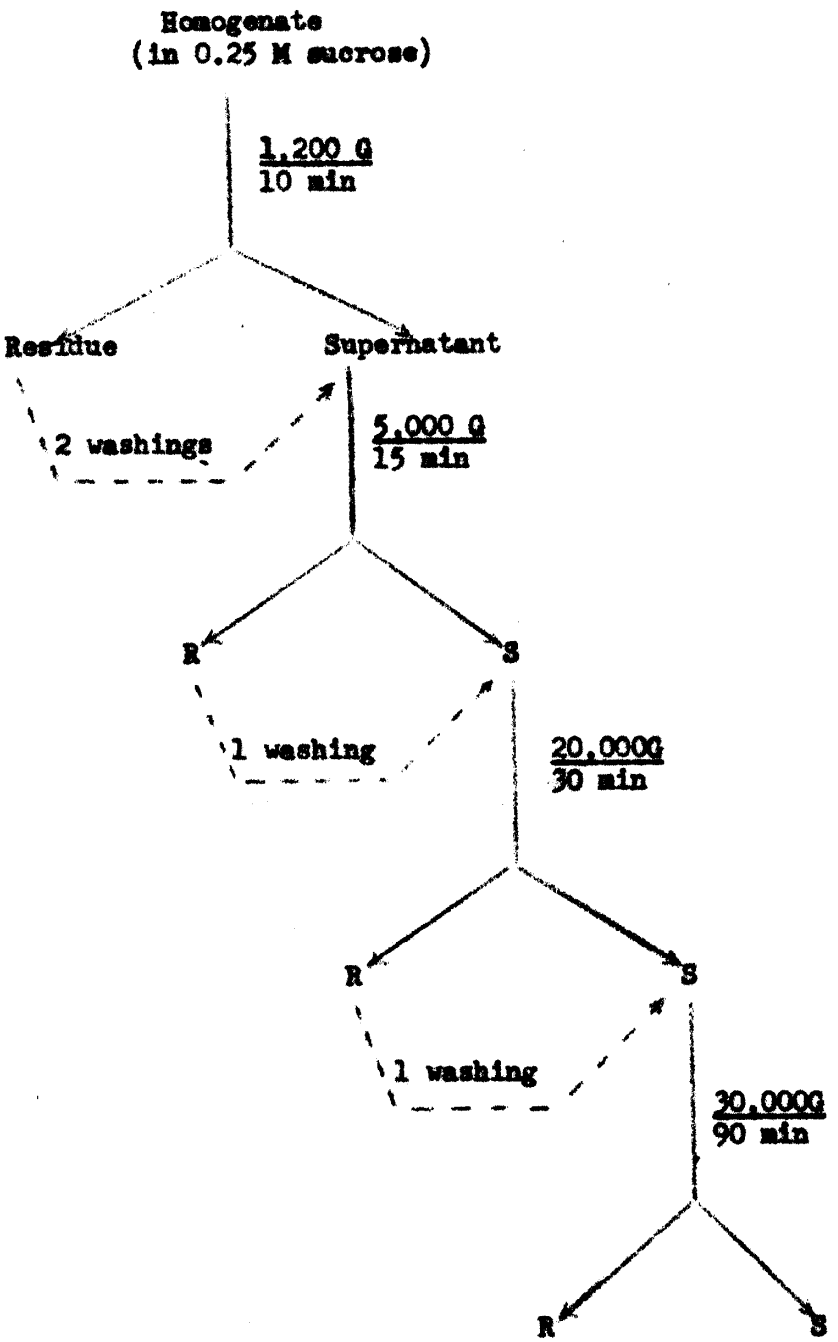

Fig. 1. Scheme of the fractionation procedure by differential centrifugation.

The centrifugations at the lowest and highest speeds were done in a IEC refrigerated centrifuge model PR-1 equipped with high speed accessories, the remaining centrifugations being done in a Servall model SS1 angle head centrifuge in the cold room. The speed of the latter was measured with the aid of an electrically driven sonometer as described below.

During centrifugation at 20,000 G or above care was taken to allow the centrifuge to lose speed gradually thus avoiding any stirring of the residues.

Measurement of Rotational Speeds. The following method was used because of its advantage in that measurement can be effected without any mechanical contact with the rotating object.

In figure 2 a permanent magnet (M) made out of a small piece of razor blade is affixed to the center of the non-magnetic lid of the centrifuge by means of adhesive tape. The magnet stays in place at maximum speed, and does not disturb the balance of the centrifuge. At the same level with the magnet a coil (C) is mounted on the cage

---

for ten minutes and a centrifugation at 5000 G for 15 minutes. Gravitational forces are expressed in thousands x gravity in the numerator, while the duration of centrifugations is expressed in minutes in the denominator. S 30/90 denotes the supernatant of a centrifugation at 30000G for 90 minutes.

of the centrifuge (not shown). These form the elements of an alternating current generator with the magnet as the rotor and the coil as the stator. The alternating current induced in the coil is led to an audiofrequency amplifier (A). The amplified current is then passed through the string (W) of a sonometer which passes between the poles (N,S) of a permanent magnet. The string, of copper wire, is fixed at one end (F_1) but free to slide over a fixed glass bridge (F_2). The other end is attached to an expansion spring (R) which can be stretched by turning a knob (K) in the form of a pulley. A pointer (P) is attached to the movable end of the spring to read over a scale (D). By adjusting the tension in the wire a point is reached when its frequency of vibration is equal to that of the alternating current generated by the rotating object. The wire is then seen to vibrate and the frequency is read off the scale.

The frequency of vibration, f, of the sonometer string is expressed as follows: $f = \frac{1}{2L}\sqrt{\frac{T}{m}}$ cycles per second; where L is the length of the wire, T, the tension of the wire, and m, its weight per unit length. Since L and m are fixed T becomes kf^2 where k is a constant that can be determined experimentally with the aid of a diapason. The tension T is found by calibrating the spring (R) on the scale (D). After determining k the scale

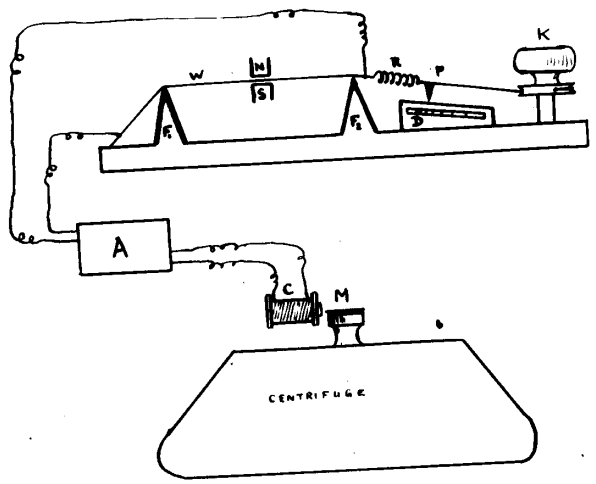

Fig. 2. Schematic diagram of the apparatus used for the mesurement of rotational speeds.

is regraduated in cycles per second. In speed measurement the reading is taken only at the point where maximal vibration of the wire is obtained. This is important in distinguishing the fundamental from the harmonics of the wire.

Although the scale of the sonometer does not reach above 220 cyc. p.s. frequencies 3 times as great can be measured by obtaining three internodes on the vibrating wire. The frequency read on the scale should then be multiplied by the number of internodes.

The accuracy of the instrument was tested stroboscopically as follows: A disc with two narrow slits along its diameter was driven by a synchroneous motor at 50 cyc. p.s. A black mark painted along the radius of the centrifuge was viewed through the slits. The eye thus glimpses at the centrifuge 100 times each second. When the speed of the centrifuge reaches a certain multiple of that of the rotating disc a stationary image is seen through the stroboscope. The results are tabulated below.

stationary figure seen through stroboscope	cycles per second. (Stroboscope)	cycles per second with sonometer.
	75	76
	100	100
	125	126

133.3	134
150	152
166.7	168
175	177
200	201

The error of the sonometer is thus a little more than 1%.

Centrifugal force can be expressed as follows: $F = 4\pi^2 n^2 r$ dynes per gram, where n is the number revolutions per second and r the distance from the rotating object to the axis of rotation. For convenience this can be expressed in terms of the earth's gravitational force: $G = \frac{4\pi^2 n^2 r}{980}$ "~~gravities~~" per gram, where 980 is the acceleration due to the earth's gravity.

Enzyme Assay. Each digest was composed of the following: 0.5 cc. enzyme, 2.5 cc. buffer, 0.5 cc. activator as $MgCl_2$ (distilled water was used when no activator was needed). The mixture was then allowed to incubate for 10 to 15 minutes before adding 1.5 cc. of the substrate. The digest was then incubated for 60 minutes at 37°C after which time the reaction was stopped by 1 cc. of 48% trichloroacetic acid (TCA). The mixture was centrifuged and inorganic phosphate was determined on aliquots of the protein-free supernatant. When assaying with adenosine 5' phosphate (A5P) one fifth of the above volumes were used.

The alkaline phosphatase was assayed at pH 9.0 (glass electrode) in the presence of 0.05M veronal

buffer, 0.01M $MgCl_2$, and 0.015M sodium beta-glycero-phosphate (Mallinckrodt). The acid phosphatase was assayed at pH 5.0 in the presence of 0.07M acetate buffer and 0.015M sodium beta-glycerophosphate. The 5 nucleotidase was assayed at pH 8.0 in the presence of 0.04M veronal buffer, and 0,0025M adenosine 5' phosphate (Nutritional Biochemicals Corp.)

Preformed inorganic phosphate in the enzyme was determined by adding 4.5 cc. of distilled water, and 1 cc. of 48% TCA to 0.5 cc. of the enzyme. The inorganic phosphate was then determined in the protein-free supernatant. Preformed phosphate was also measured in the substrate medium composed of 1.5 cc. substrate solution, 0.5 cc. $MgCl_2$ (0.1M), 2.5 cc. buffer, 0.5 cc. distilled water instead of enzyme, and 1 cc. of 48% TCA.

Activity units are expressed as mg. of P liberated per hour at 37°C, and the specific activity as µg P liberated per hour per mg N.

Inorganic Phosphate was determined by the method of Fiske and Subbarrow (19) modified for use with the Junior Coleman Spectrophotometer at a wavelength of 660 mµ. Standards of known P concentration and blanks were prepared containing the same amount of 8% TCA found in the unknown sample so that Molybdate II could be used for all determinations.

Total Nitrogen was determined by a micro Kjeldahl method (20,21) with direct titration of the liberated ammonia (22).

.. ..

DESCRIPTION OF THE PARTICULATE FRACTIONS

General Description. The first fraction obtained by centrifugation G 1.2/10 has a dirty white appearance with a red patch of packed erythrocytes at its bottom. Microscopically examined it is found to be composed of nuclei, erythrocytes, tissue fragments, and unbroken cells. Fraction 1.2/10 G 5/15 is milky white in appearance. Under the microscope are seen spheroidal particles ranging in size from 1 micron or less to 4.5 micra.* An occasional nucleus may be found. Fraction 5/15 G 20/30, also white, is composed of particles less than 1 micron in size. Fraction 20/30 G 30/90 is a transparent gelly-like pellet of a faint rose color. More vigorous shaking is required to resuspend it than for the previous fractions. It consists of particles smaller than those of the preceding fraction; their size cannot be measured with an ordinary light microscope.

Staining Properties. The dye Janus Green B which is known as a specific supravital stain for mitochondria at low concentrations (23,24) was applied to the various particles in an experiment similar to that described by Recknagel (25) on liver tissue. It appears that the dye

---

* As measured with an ocular micrometer scale.

is reduced to a pink compound by the respiratory enzymes which are found mostly in the mitochondria. This criterion, as Recknagel et al suggest, may be used in turn to identify particulate material as mitochondria. The dye was therefore tested on the brain particles to find out which fraction would change the color of the dye.

Janus Green B (Coleman & Bell Co.) was dissolved at a concentration of 1:20,000 in 0.25M sucrose solution. This mixture was used as a suspending medium for homogenization, centrifugation, and washing of the various fractions. The differential centrifugation procedure was carried out as described previously. All the particles thus obtained adsorbed the dye to the extent that the supernatant and washings were nearly colorless. The phenomenon resembles the adsorption of colored substances by charcoal. Fraction 20/30 G 30/90, still a transparent pellet, was stained blue like the previous fractions.

The brain particles behaved like those of liver in that the dye was changed to an intense pink when warmed to 37°C. The results were as follows:

	G	1.2/10	: no significant change in color
1.2/10	G	5/15	: changed to intense pink in about 5 minutes. The change was noticeable even before warming.
5/15	G	20/30	: changed to intense pink in about 15 minutes.
20/30	G	30/90	: no change in color.

Presumably the change in color in the brain particles may also be due to respiratory enzymes as in the case of liver tissue. Fractions 1.2/10 G 5/15 and

5/15 G 20/30 which change in color, therefore, contain the respiratory enzymes and would accordingly be classified as mitochondria. The remaining cytoplasmic particles (20/30 G 30/90) would then correspond to the microsomes.

.. ..

DISTRIBUTION OF THE ACID AND ALKALINE PHOSPHATASES

Acid Phosphatase. The study of the distribution of the phosphatases in brain was done in a series of four major runs each comprising differential centrifugation, enzymatic assay, and determinations of total nitrogen for measurement of specific activities. The distribution of acid phosphatase in the various particulate fractions is shown in tables I to IV inclusive. It is seen that about 90 percent of the enzyme activity is present in the cytoplasm (tables II to IV). the enzyme may, therefore, be considered essentially cytoplasmic. The specific activity of the enzyme is, however, nearly the same in the nucleus and in the cytoplasm since the nucleus contains only about 10 to 16 percent of total nitrogen of the cell (table IX).

In experiment I the nuclear fraction, G 1.2/10, was insufficiently washed (one washing instead of the usual two). As a result the enzyme activity and the nitrogen content (table IX) are relatively higher than in the other experiments. This is most probably due to cytoplasmic contaminants. The results of this experiment were retained to show the effects of washing by resuspension and recentrifugation of the particles.

Fractions 1.2/10 G 5/15 and 5/15 G 20/30 are the richest of the cytoplasmic particles in acid phosphatase. These two fractions also behave alike with respect to the dye Janus Green. This suggests that the

majority of the enzyme activity is found in what may be the mitochondria.

The enzyme content of the final supernatant, S 30/90, shows wide variations and seems to follow the nitrogen content of that fraction. This is due to the amount of mechanical damage done to the particles, and will be presently discussed.

Alkaline Phosphatase. The distribution of this enzyme was determined in the same four experiments mentioned above and is shown in tables V to VIII inclusive. It is seen that the nuclear fraction is richer than any other fraction in alkaline phosphatase activity. The specific activity shows more clearly the relative concentrations of the enzyme in each fraction. The nuclear fraction consistently has the highest specific activity while the fraction coming next after it, 1.2 G 5/15, has the lowest specific activity. Fraction 20/30 G 30/90 shows another peak in the specific activity of the fractions suggesting the probable presence of another isodynamic alkaline phosphatase. The nuclear fraction of experiment I shows a relatively high activity, also the result of improper washing.

It is seen also that the activity of alkaline phosphatase usually increases upon standing in the refrigerator. In experiments II and IV all the fractions had increased in activity after standing a few days. The specific activities were, therefore, calculated from the

higher activities since these are more representative of the true enzyme content of the fraction.

.. ..

TABLE I - Distribution of Acid Phosphatase in Experiment I.

The enzyme was assayed at pH 5.0 in the presence of 0.015M sodium B-glycerophosphate and 0.07M acetate buffer. The activities were determined as mg P liberated per hr. per cc. of enzyme. This value was then multiplied by the volume of the fraction to find its total activity. The same conditions apply for the results shown in Tables II to IV.

Fraction	1st day after fractionation				16th day
	mg P liberated per hr.by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberat per hr. by fraction
G 1.2/10	4.22	30.4	165.5	25.5	5.15
1.2/10 G 5/15	4.02	28.9	75.5	53.2	4.35
5/15 G 20/30	2.24	16.1	19.4	115.5	2.19
20/30 G 30/90	0.87	6.24	13.85	62.6	1.52
S 30/90	2.57	18.5	67.0	38.4	2.13
Original Homog.	13.9	Total 100.1	353.0	39.4	17.9

TABLE II - Distribution of Acid Phosphatase in Experiment II

Fraction	1st day after fractionation				9th day
	mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberated per hr. by fraction
G 1.2/10	1.34	10.2	26.1	51.4	1.58
1.2/10 G 5/15	2.81	21.4	58.4	48.1	2.66
5/15 G 20/30	2.39	18.2	39.7	60.2	1.99
20/30 G 30/90	1.61	12.3	31.8	50.7	1.65
S 30/90	4.46	34.0	84.0	53.1	3.33
Original Homog.	13.1	Total 96.2	246.0	53.3	12.53

TABLE III - Distribution of Acid Phosphatase in Experiment III.

Fraction	1st day after fractionation				4th day
	mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberated per hr. by fraction
G 1.2/10	1.78	10.5	60.6	29.4	1.76
1.2/10 G 5/15	5.65	33.4	111.2	50.8	4.78
5/15 G 20/30	3.96	23.4	69.7	56.7	4.03
20/30 G 30/90	1.47	8.7	28.0	52.5	1.18
S 30/90	2.72	16.1	97.4	28.0	1.9
Original Homog.	16.9	Total 92.0	377.5	44.8	15.95

TABLE IV - Distribution of Acid Phosphatase in Experiment IV.

Fraction	1st day after fractionation				4th day
	mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberated per hr. by fraction
G 1.2/10	1.56	9.4	38.85	40.1	1.55
1.2/10 G 5/15	5.04	30.2	86.2	58.5	4.70
5/15 G 20/30	3.73	22.4	55.0	67.8	3.71
20/30 G 30/90	1.33	8.0	17.0	78.2	1.24
S 30/90	2.26	13.6	68.5	33.0	1.70
Original Homog.	16.65	Total 83.6	273.5	61.0	14.25

TABLE V - Distribution of Alkaline Phosphatase in Experiment I.

The enzyme was assayed at pH 9.0 in the presence of 0.015M sodium B-glycerophosphate, 0.01M $MgCl_2$, and 0.03M veronal buffer. The activities were determined as mg P liberated per hr. per cc. of enzyme. This value was then multiplied by the volume of the fraction to find its total activity. The same conditions apply for the results shown in Tables VI to VIII.

Fraction		2nd day after fractionation				24th day
		mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberat per hr. by fraction
	G 1.2/10	14.9	51.8	165.5	90.0	16.85
1.2/10	G 5 /15	1.315	4.56	75.5	17.4	1.04
5/15	C 20/30	0.672	2.33	19.4	34.6	0.446
20/30	G 30/90	1.295	4.5	13.85	93.5	1.17
	S 30/90	2.22	7.7	67.0	33.2	1.90
Original Homogen.		28.8	Total 70.9	353.0	81.6	29.1

TABLE VI - Distribution of Alkaline Phosphatase in Experiment II.

Fraction	9th day after fractionation				1st day
	mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberated per hr. by fraction
G 1.2/10	8.16	38.5	26.1	312.0	5.08
1.2/10 G 5/15	1.42	6.7	58.4	24.3	1.25
5/15 G 20/30	1.377	6.5	39.7	34.7	0.92
20/30 G 30/90	2.16	10.2	31.7	68.2	1.10
S 30/90	5.38	25.4	84.0	64.0	3.64
Original Homog.	21.2	Total 87.2	246.0	86.2	12.60

TABLE VII - Distribution of Alkaline Phosphatase in Experiment III.

Fraction		1st day after fractionation				4th day
		mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberated per hr. by fraction
	G 1.2/10	14.35	49.4	60.6	237.0	12.0
1.2/10	G 5/15	2.68	9.2	111.2	24.1	2.26
5/15	G 20/30	2.99	10.3	69.7	42.9	2.77
20/30	G 30/90	3.42	11.75	28.0	122.1	2.91
	S 30/90	5.18	17.8	97.4	53.2	3.42
Orig. Homogenate		29.1	Total 98.3	377.5	77.2	24.25

TABLE VIII - Distribution of Alkaline Phosphatase in Experiment IV.

Fraction	4th day after fractionation				1st day
	mg P liberated per hr. by fraction	percent of Homogenate	mg total N in fraction	Specif.activ. (µg P/hr/mgN)	mg P liberated per hr. by fraction
G 1.2/10	7.56	31.5	38.85	194.5	6.70
1.2/10 G 5/15	2.01	8.36	86.2	23.3	1.74
5/15 G 20/30	2.05	8.52	55.0	37.3	1.84
20/30 G 30/90	2.11	8.7	17.0	124.0	1.83
S 30/90	2.54	10.6	68.5	37.1	3.19
Original Homog.	24.0	Total 67.7	273.5	88.0	22.1

THE DISTRIBUTION OF TOTAL NITROGEN AND THE EFFECT OF MECHANICAL DAMAGE

The total nitrogen of each fraction obtained by centrifugation was measured in order to calculate the specific activity of that fraction. Table IX shows the nitrogen content of these fractions. The total nitrogen in the original homogenate was also determined to show the general recovery.

As mentioned before, the nuclear fraction which was washed only once (Exp. I) was found to be relatively richer in nitrogen, probably due to cytoplasmic contaminants. In the other three experiments where the nuclear residue was washed twice, this fraction still contains an appreciable amount of contaminating material as shown by microscopic examination. A clear cut separation of the nuclei from the cytoplasm seems difficult if not impossible without some loss of either component. Judging from the other three experiments it appears, therefore, that the cytoplasm contains at least 84 percent of the total nitrogen of the cell.

The total nitrogen of the cytoplasmic particles, on the other hand, appears to be greater the lower the centrifugal speed at which they were obtained (i.e. the larger the particles). The final supernatant, however,

TABLE IX - Distribution of Total Nitrogen in the Brain Fractions Obtained by Centrifugation.

Experiment No.		I		II		III		IV	
Fraction		mg total N	percent of Homogenate	mg total N	percent of Homogenate	mg total N	percent of Homogenate	mg total N	percent of Homog.
	G 1.2/10	165.5	46.9	26.1	10.6	60.6	16.05	38.85	14.2
1.2/10	G 5/15	75.5	21.4	58.4	23.7	111.2	29.5	86.2	31.5
5/15	G 20/30	19.4	5.5	39.7	16.25	69.7	18.45	55.0	20.1
20/30	G 30/90	13.85	3.9	31.8	12.9	28.0	7.4	17.0	6.2
	S 30/90	67.0	19.0	84.0	34.1	97.4	25.8	68.5	25.1
Original Homog.		353.0	96.7	246.0	97.5	377.5	97.4	273.5	97.1

shows an inconsistant nitrogen content. An explanation for this odd result was suggested by de Duve et al (26,27). They find that the mitochondria of liver lose part of their protein content (acid phosphatase) into the suspending medium upon rough treatment. The following experiment was, therefore, devised to test this on brain particles.

One cerebral hemisphere was removed from a dog under anesthesia and immediately homogenized in a Potter-Elvehjem tube in the cold room with about an equal volume of 0.25M sucrose. More sucrose solution was added to make a suspension of 1:5 (w/v) which was strained from debris through several layers of flannel gauze. This Potter-Elvehjem homogenate was divided into two aliquots one of which was subjected to the action of the Waring blendor for 5 minutes in the cold (1 minute at a time to prevent heating). The resulting homogenate, WH, was diluted 2.5 times* with sucrose solution resulting in a diluted homogenate, W. The other aliquot of the Potter-Elvehjem homogenate was also diluted 2.5 times resulting in a suspension, E. This was further subdivided into two aliquots, one of which was also subjected to the Waring blendor for 5 minutes giving a suspension EW. The other aliquot of E together with aliquots of W and EW were centrifuged at 30,000 G for 90 min yielding supernatants SE,SW, and SEW respectively. The residues were discarded. Figure 3 represents a scheme of the experiment.

The fractions thus obtained were assayed for enzyme activity on the same day of the experiment. Table X shows the enzyme concentrations in the fractions as well as the total N / conte

* This dilution gives a supernatant of about the same concentration in total nitrogen as that of S 30/90.

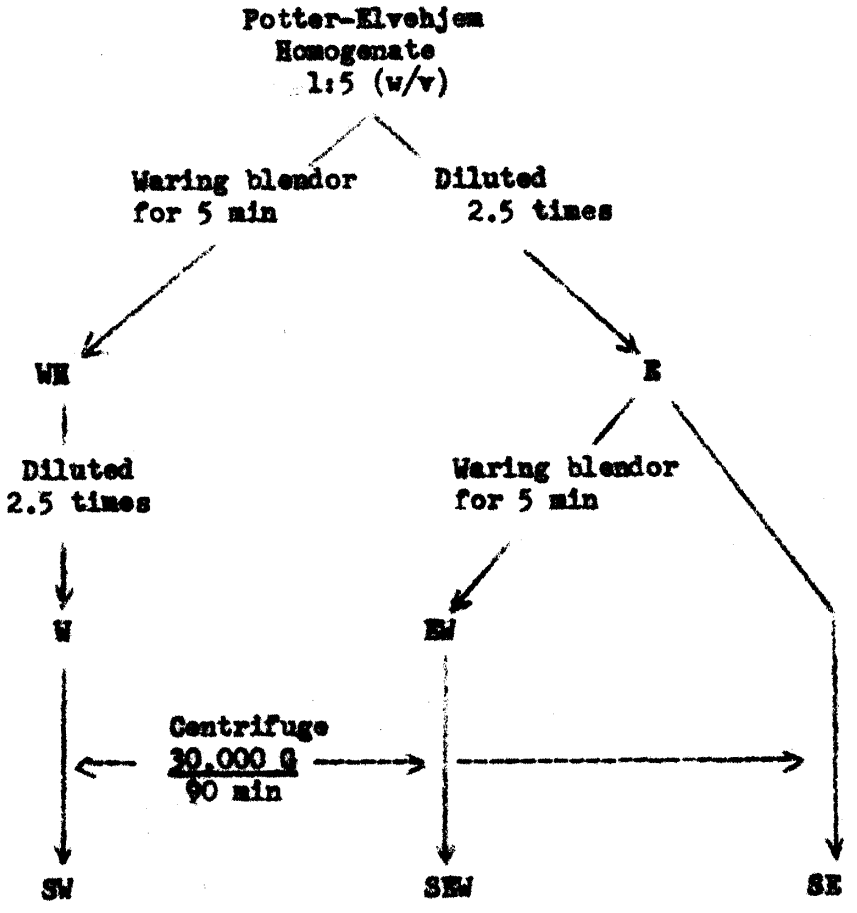

Fig. 3. Scheme of the experiment to show the effect of mechanicql damage by the Waring blendor. The suspending medium is 0.25 M sucrose.

TABLE X - The Action of the Waring Blendor

Enzyme activities are expressed as mgP liberated per hr. per cc. enzyme suspension. See figure 3.

Fraction	mg total N per cc.	Inorganic phosphate mgP per cc.	Acid Phosphatase	Alkaline Phosphatase
E	-	0.0155	0.0483	0.0455
W	0.731	0.0218	0.0476	0.0382
EW	0.725	0.0230	0.0519	0.0390
SW	0.206	0.0152	0.0264	0.0097
SEW	0.181	0.0163	0.0275	0.0093
SE	0.181	0.0146	0.0096	0.0081

The results show that the concentrated homogenate (1:5 suspension) is more sensitive to mechanical damage administered by the Waring blendor blades than is the diluted homogenate. This is evidenced by the fact that the final supernatant SW has a higher concentration of total N than the supernatants SEW and SE. Fractions W and EW theoretically should have the same concentration of total N, the difference is due to experimental error. Since mechanical damage during homogenization cannot be controlled these results may explain the wide differences in the nitrogen content of the supernatants S 30/90 in the four experiments on the fractionation of cerebral tissue. The same Potter-Elvehjem tube was used through all experiments for the sake of uniformity.

That the enzyme concentration of S 30/90 is also affected by mechanical damage was demonstrated by the same experiment. Table X shows that the acid and alkaline phosphatases of the supernatant fluid is of a greater concentration in the supernatants coming from a homogenate subjected to the Waring blendor. The acid phosphatase appears to be more sensitive to such treatment than the alkaline phosphatase. It is seen that the acid enzyme is actually activated by mechanical damage when treated in the diluted suspension, EW. When the concentrated homogenate is "blended" before dilution both enzymes appear to be slightly inactivated as compared to the activity in E. The cause of this inactivation has

not been investigated. It may be that the blades of the blendor are releasing traces of inactivating metal (28), or that the mechanical damage by surface denaturation itself inactivates these enzymes which are proteins. Mechanical injury may explain why incomplete recoveries are obtained, especially so with the alkaline phosphatase.

Another experiment was undertaken to find out whether dilution has any effect on the concentration of total N in the final supernatant.

As before, cerebral tissue was homogenized in the cold in a Potter-Elvehjem tube and a 1:5 suspension was made thereof with 0.25M sucrose solution. Two aliquots of this suspension were used as follows:

a. The concentrated 1:5 homogenate was diluted twice with 0.25M sucrose

b. The concentrated homogenate was diluted 3 times with 0.25M sucrose

(a) and (b) were allowed to stand for 2 hrs. in the cold room. An aliquot of each was then centrifuged at 30,000G for 90 min yielding supernatants SA and SB. Figure 4 summarizes the experiment. The results are shown in table XI.

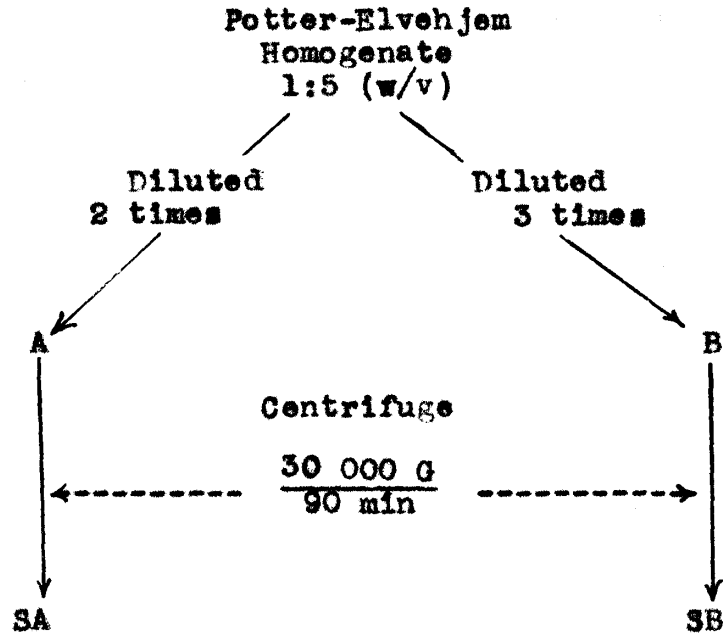

Fig. 4. The effect of dilution on the soluble nitrogen in brain homogenate.

TABLE XI. The solubility of total nitrogen in the final supernatant fluid.

Fraction	mg total N per cc.	Factor	Product (or equivalence) mg N
A	1.228	x 2	2.46
B	0.835	x 3	2.50
SA	0.330	x 2	0.66
SB	0.233	x 3	0.697

The results show that the more dilute the suspension the more the amount of total nitrogen "dissolved" in the final supernatant (probably in the form of proteins). Thus the amount of total N in S 30/90 depends partly on its final volume. This may be another reason for the wide variations of total nitrogen in the final supernatants, S 30/90. In experiment I which had the least number of washings the supernatant has the lowest percentage of total N.

.. ..

ON THE ACTIVITY OF 5-NUCLEOTIDASE

The Measurement of 5-Nucleotidase Activity. The assay of the specific enzyme 5-nucleotidase presents a difficulty in that its substrate (5' phosphoric esters of nucleorides) is also hydrolyzed by the non-specific alkaline and acid phosphatases. Thus the inorganic phosphate liberated by digesting a 5' phosphoric nucleoside with a mixture of these enzymes will not be due to the activity of 5-nucleotidase alone, nor will it represent the sum of the activity of these enzymes because of possible competition for the substrate. Since the acid phosphatase acts at an optimum pH of 5.0, while the 5' nucleotidase acts within the range 7.5 to 8.5 (8,29), the effect of the acid phosphatase within these limits will be negligable. The non specific alkaline phosphatase, however, is expected to dephosphorylate nucleosides quite readily since its optimum pH of 9.0 is not far from pH 8 - the optimum for 5' nucleotidase.

To estimate the true activity of 5 nucleotidase Reis (7,8), who first reported its presence in brain, compared the rates of hydrolysis of adenosine 5' phosphate and of β-glycerophosphate. Later, in an estimation of the 5-nucleotidase activity of various human tissues (30), the same author deduced the activity of the enzyme " by measuring the activity against adenylic acid and subtracting that measured against some other phosphoric ester" (eg., phenyl phosphoric acid). This method, however, is valid

only if the non-specific phosphatase hydrolyzes both substrates at the same rate, and if competitive effects are neglected. Reis makes no mention as to whether this is the case or not.

Heppel and Hilmoe (29), who later purified 5-nucleotidase from bull seminal plasma, found that the enzyme was heat-stable (20 minutes at 60°C) while the non-specific alkaline phosphatase lost most of its activity under these conditions. It appears, therefore, that by subjecting a preparation to heat one should be able to measure the 5-nucleotidase activity in the absence of other interfering activities.

Our preliminary experiments showed that the activity of 5-nucleotidase decreases gradually when heated, but at a much slower rate than the non-specific alkaline phosphatase. A study of the decrease in activity with time showed that it is a reaction of the first order, the equation being

$$A_O = Ae^{-kt}$$, where

A_O = initial activity

A = activity at time t.

k = a constant dependent on the nature of the enzyme and on the temperature at which it is being destroyed.

The initial activity can be estimated by plotting the logarithm of A against time, the relationship being

linear. The value of logarithm A_o is then obtained by extrapolation to the ordinate.

It was found that the hydrogen ion concentration has no significant effect on the rate of denaturation of the enzyme between pH 5.5 and 7.5. Beyond these values denaturation is more rapid.

Figure 5 summarizes the results of an experiment on the denaturation of 5-nucleotidase by heat. Cerebral tissue (dog) was homogenized in the cold in a Potter-Elvehjem tube in 0.25M sucrose, making a 1:5 (w/v) suspension of the original tissue. The mixture was then placed in a water bath at 60°C, samples being removed at known intervals for enzyme assay and chilled in ice. The graph (Fig.5) shows the results immediately after heat treatment, and also two days subsequent to such treatment (the samples being stored in the refrigerator). Activity is expressed in mg.P liberated per hour per cc. of enzyme suspension. The relationship between the logarithm of activity and time appears to be linear only within the first two hours, after which time the reaction behaves erratically. The activity of the alkaline phosphatase on the same preparation is summarized in table XII.

Now, it was found that the activity of the unheated mixture containing both the non-specific phosphatases and 5-nucleotidase was always less than the value obtained after heating and extrapolating to zero time. If both enzymes were acting simultaneously the activity of the unheated

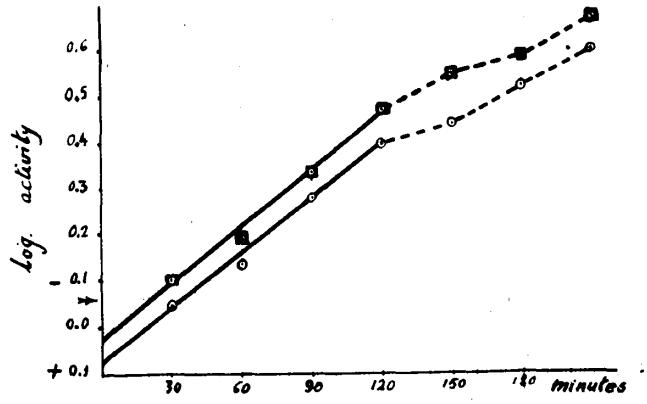

Fig.5: Destruction of 5-nucleotidase at 60°C at pH 6.0. Activities are expressed as mg P liberated per hr. per cc. enzyme when assayed at pH 8.0 in the presence of 0.04M veronal buffer, 0.01M $MgCl_2$, and 0.0025M A5P at 37°. The arrow indicates the value for the activity of the unheated aliquot. Symbol (⊙) denotes the logarithm of activities immediately after heat treatment, and symbol (⊡) two days later. The same conditions hold for figures 6,8,9,10,11,12 and 13.

TABLE XII. Alkaline Phosphatase Activity of the same samples whose 5-nucleotidase activity is given in fig.5. The conditions of assay are the same as those given for table V.

Minutes at 60°C	mg P liberated per hr. per cc.	percent of unheated sample
0	0.298	100
30	0.018	6.1
60	0.015	5.1
90	0.013	4.5

mixture should be greater than after heating. If, on the other hand, the non-specific phosphatases were absent to begin with the extrapolated zero value and that of the unheated mixture should be identical.

The fact that the extrapolated zero value for the activity of 5-nucleotidase is higher than the unheated sample may be explained by one of the following two hypotheses:

1) A natural inhibitor present with the 5-nucleotidase is destroyed by heat faster than the enzyme.
2) A portion of the enzyme is present in a bound inactive form and is released by heat.

It is of course possible that both explanations may apply at the same time.

No attempt was made to test the validity of the first hypothesis. The second presents several possibilities, one of them being that the enzyme may be bound within particles having an outer membrane, thus releasing the enzyme.

To test this possibility a brain suspension was made in distilled water instead of isotonic sucrose solution and it was subjected to mechanical damage through the action of the Waring blendor for 5 minutes in the cold. The activity of the unheated enzyme suspension was now found to be almost the same as the extrapolated zero value obtained by heating at 60°C. If all the enzyme had been released, a higher value should have been observed for the

unheated preparation because it contains also the non-specific phosphatase. Since this was not the case, one may conclude that a portion of the enzyme is bound to a protein in an inactive form (a condition similar to that found for trypsinogen and trypsin). The possibility that the enzyme is confined within a particle with an outer membrane is ruled out by the fact that the final supernatant fluid, S 30/90, also shows an apparent activation by heat treatment (Fig. 12).

That the activity of 5-nucleotidase actually first increases and then decreased during the first twenty minutes at 60°C was demonstrated in another experiment. The results are shown in Fig. 6 and indicate that the higher value upon extrapolation to zero time is not due to a latent period before the onset of denaturation but that heating actually causes an increase in activity.

The alkaline phosphatase was also measured in the same aliquots taken for 5-nucleotidase assay. The results (Fig. 7) show that the alkaline phosphatase behaves similarly to 5-nucleotidase, the extrapolated zero value being higher than that of the original unheated sample. The activity of the enzyme drops to a low value within 25 minutes then remains practically constant on further heating. Table XIV shows the activity of the heated alkaline phosphatase in the

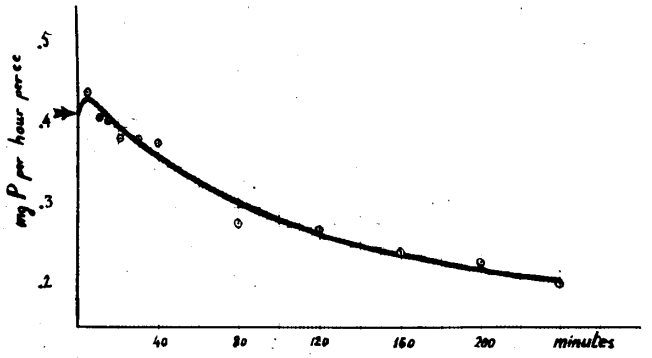

Fig. 6. Destruction of 5-nucleotidase at 60°C at pH 6.7 . Measurements of enzyme activity were done at short intervals in the first stages of heating at 60°C. The hump in the curve within the first 20 min of heating shows that the enzyme is slightly activated by heat during the denaturation process. The arrow points at the logarithm of the activity of the unheated sample. Fig. 7 gives the alkaline phosphatase activity of the samples in the same experiment.

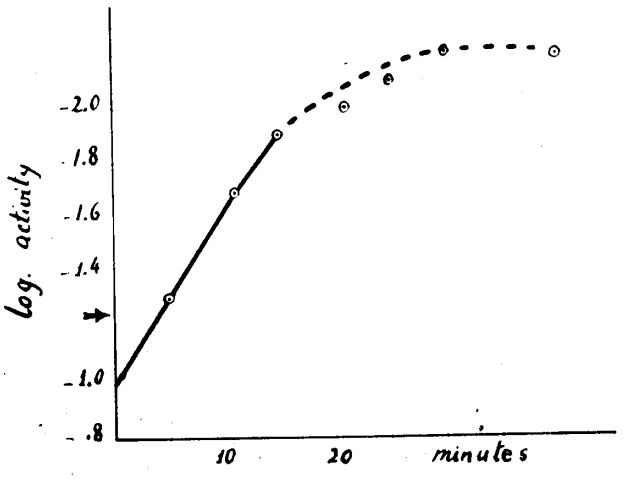

Fig. 7. Destruction of alkaline phosphatase at 60°C at pH 6.7 Activities are expressed as mg P liberated per Hr. per cc of enzyme when assayed at pH 9.0 in the presence of 0.03M veronal buffer, 0.01M $MgCl_2$, and 0.015M Na_2 B-glycerophosphate at 37°C. The arrow points at the value of the activity of the unheated aliquot. At 30 min. there remains only 9.2% of the activity of the unheated aliquot, or 6.1% of the value found upon extrapolation to zero time.

various fractions of experiment III. The results seem to indicate that, irrespective of the initial concentration, the low activity reached after heating is practically the same in all fractions.

The Distribution of 5-Nucleotidase. The above method of measuring the "true" activity of 5-nucleotidase was applied to a study of the distribution of this enzyme in a brain homogenate in which acid and alkaline phosphatase were also determined (Experiment III). Table XV shows the activity of the "raw" fractions on A5P. An aliquot of about 50 cc. of each fraction (pH 6.7-7.0) was then heated in a water bath at 60°C, samples being removed at regular intervals for enzyme assay. The assay was made immediately after heating, since the activity decreases upon standing subsequent to heat treatment (see Fig.5). It is interesting to note, however, that this is not the case with the unheated enzyme (Table XV). This tends to suggest that the usual slight activation in the first stages of heating is merely a hastening of the process of autolysis.

The results of these experiments are shown in figures 8 to 13 inclusive. The logarithm to the base 10 of A_0 of each fraction found by extrapolation to zero time is given in table XIII. The antilogarithm thus represents the activity in mg P liberated per hour per cc. of suspension. This value multiplied

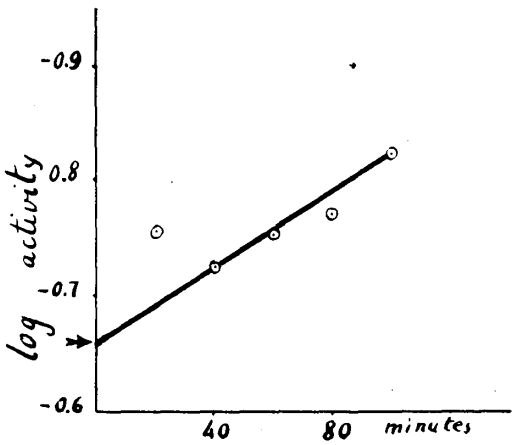

Fig. 8. Destruction of 5-nucleotidase in fraction Gl.2/10 (nuclear fraction) . Activity by extrapolation to zero time = 0.220 mg P liberated per hr. per cc of enzyme.

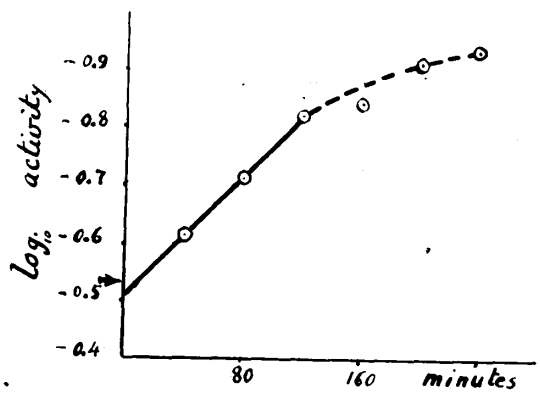

Fig. 9. Destruction of 5-nucleotidase in fraction 1.2/10 G 5/15. Initial activity by extrapolation to zero time = 0.309 mg P liberated per hr. per cc of enzyme.

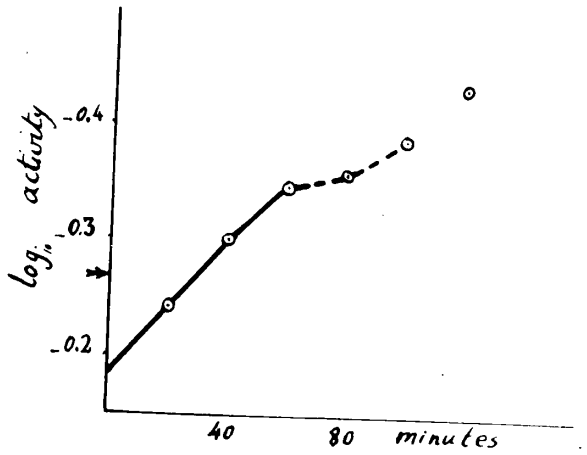

Fig. 10. Destruction of 5-nucleotidase in fration 5/15 G 20/30. Initial activity by extrapolation to zero time = 0.653 mg P liberated per hr. per cc of enzyme.

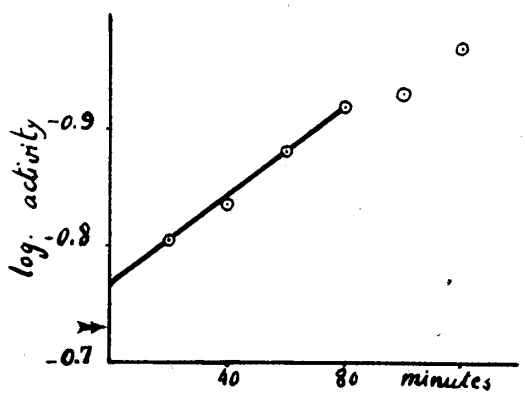

Fig. 11. Destruction of 5-nucleotidase in fraction 20/30 G 30/90. Initial activity by extrapolation to zero time = 0.171 mg P liberated per hr per cc of enzyme.

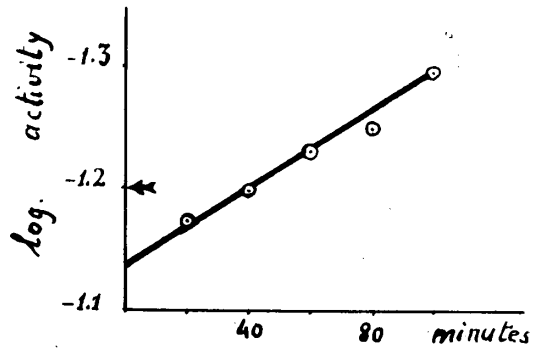

Fig. 12. Destruction of 5-nucleotidase activity in fraction S30/90. Initial activity by extrapolation to zero time = 0.0729 mg P liberated per hr. per cc of enzyme.

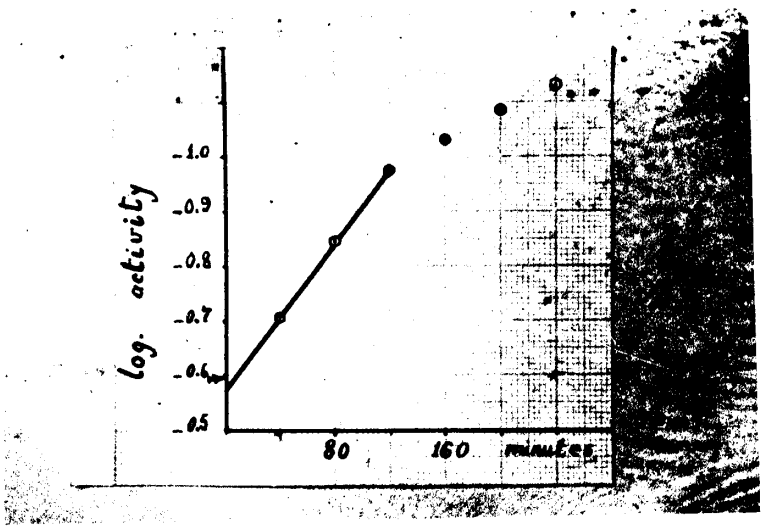

Fig. 13. Destruction of 5-nucleotidase in the original homogenate. Initial activity by extrapolation to zero time = 0.266 mg P liberated per hr. per cc of enzyme.

TABLE XIII - The Activity of "Purified" 5-Nucleotidase

The enzyme was assayed at pH 8.0 in the presence of 0.0025M A-5-P, 0.01M $MgCl_2$, 0.04M veronal buffer, and was incubated for 1 hr. The total activity of each fraction is calculated by multiplying the activity per cc. (antilog.) by the final volume of each fraction. (See figures 8 to 13 for values of logarithms).

Fraction			logarithm to base 10 of activity (by extrapolation)	Antilogarithm or mg P/hr/cc	mg P liberated per hr. by fraction	percent of Homogenate	Specif.activ. (µg P/hr/mgN)
	G	1.2/10	-0.657	0.220	13.2	10.3	218
1.2/10	G	5/15	-0.510	0.309	21.63	16.9	194
5/15	G	20/30	-0.185	0.653	39.2	30.4	563
20/30	G	30/90	-0.767	0.171	25.1	19.6	897
	S	30/90	-1.137	0.0729	23.0	18.0	236
Original Homog.			-0.575	0.266	128.0	Total 95.4	339

TABLE XIV - Alkaline Phosphatase Activity on the same Brain fractions shown in tables XII, XIII and XV (experiment III).

Fraction	Unheated µg P/hr/cc	Heated µg P/hr/cc	percent of unheated fraction
G 1.2/10	239.0	3.6 after 20 min.	1.5
1.2/10 G 5/15	38.3	4.1 " 40 "	10.8
5/15 G 20/30	49.8	4.5 " 40 "	9.04
20/30 G 30/90	23.3	6.3 " 20 "	37.0
S 30/90	16.4	3.6 " 40 "	21.9
Original Homog.	60.6	3.3 " 40 "	5.45

TABLE XV - 5 Nucleotidase activity of the various unheated fractions of brain homogenate. The conditions are the same as those given under Table XIII.

Fraction	mg P liberated per hr. per cc.	logarithm to base 10	Volume of fraction cc	mg P liberated per hr. by fraction (1st day)	percent of Homogenate	mg P liberated per hr. by fraction (5 days later)
G 1.2/10	0.219	-0.659	60	13.15	10.8	14.2
1.2/10 G 5/15	0.294	-0.532	70	20.6	16.9	20.7
5/15 G 20/30	0.539	-0.269	60	32.35	26.7	32.2
20/30 G 30/90	0.186	-0.73	147	27.4	22.5	29.8
S 30/90	0.0631	-1.20	316	19.9	16.3	26.9
Original Homog.	0.2538	-0.596	480	121.8	Total 93.3	136.8

by the volume of the fraction gives its total activity.

The percentage distribution of 5-nucleotidase (table XIII) throughout the fractions is more uniform than that of the acid and alkaline phosphatases. The specific activity of the fractions, however, shows that the 5-nucleotidase concentration is inversely proportional to the size of the cytoplamsic particles, the highest value being found in fraction 20/30 G 30/90.

The nuclear fraction has a higher specific activity than that of the fraction coming next after it. Unlike the case of acid phosphatase this tends to show that the nuclei do have some 5-nucleotidase activity of their own, and that the higher value could hardly have resulted from contamination of the nuclear fraction with part of fraction 1.2/10 G 5/15.

In general the results show that the percentage distribution of "purified" 5-nucleotidase is not very different from that of the "raw" fractions containing also the non-specific alkaline phosphatase. This indicates that the latter enzyme is not very active under the conditions of assay for 5-nucleotidase.

Fraction 20/30 G 30/90 is exceptional in that the activity of 5-nucleotidase found by extrapolation to zero time is less than that of the "raw" fraction (Fig. 11). This may be explained by the loss of alkaline phosphatase activity, there being very little 5-nucleotidase present in

the "bound" state.

.. ..

DISCUSSION

The Effect of Anesthetics. The only work with any bearing on the effect of anesthesia on the acid and alkaline phosphatases was done on dog's cerebral cortex by Swank and Cammermeyer (33). Their semiquantitative histochemical study shows that the acid and alkaline phosphatases exhibit oscillatory changes in activity over a period of 7 hours, and that these changes are dependent not on the depth but on the duration of narcosis. The anesthetics used were sodium amytal and ether. Since the control brains they used were obtained by killing the animal immediately after anesthesia, it is impossible to tell as yet what are the immediate effects of anesthesia on these enzymes.

Homogenization. The Potter-Elvehjem tube (18) was chosen as the instrument of homogenization rather than the Waring blendor. Its advantages are that it has no metal parts and that it does not damage the nuclei or mitochondria. These advantages are discussed by Schneider and Hogeboom in a review on the isolation of cell components (11). Berthet et al (27) have also shown the need of avoiding unnecessary mechanical damage when dealing with such systems as the acid phosphatase of mitochondria. They also suggest for this reason that the number of washings be reduced to a minimum during the fractionation procedure. The detrimental effect of the Waring blendor on alkaline phosphatase as well, and the possible errors

in distribution that would ensue have been demonstrated by the data in table X. The only occasion where the shearing action of the Waring blendor blades can be made use of is probably in the isolation of chromosomes (34).

The necessity of avoiding electrolytes in the suspending medium has been ddscussed by Schneider and Hogeboom (11). Clumping of particles occurs when electrolytes are present in the medium. Isotonic sucrose (0.25M) has been claimed as the most preferable medium for fractionation studies on the intracellular distribution of enzymes by the same authors as well as by Bebthet et al (14) and Brody and Bain (16). Isotonic sucrose was therefore selected as the suspending medium for homogenization and centrifugation.

Differential Centrifugation and the Isolation of Cytoplasmic Granules. Differential centrifugation has been used extensively in the isolation of mitochondria and other cell particles ever since the technique was introduced by Bensley and Hoerr (9). Yet, even when the same suspending medium was used different centrifugal speeds were chosen by different authors for the isolation of what they all name the mitochondria and microsomes of the cell.

Chantrenne (17), on the other hand, has shown that one can obtain a continuous array of particles of a wide range in size with an equally wide range of centri-

fugal speeds, and that there exists no sharp dividing line in the size of the "large granules" and the "microsomes". In spite of what Chantrenne has clearly demonstrated, different authors have continued to use different speeds for the isolation of these cell components. Table XVI lists a few instances to show the diversity of conditions under which these operations were carried out. The terms "mitochondria" and "microsomes", for this reason, have been used non-commitally throughout this text. The centrifugal speeds in the present study were also chosen more or less arbitralily.*

Fraction 1.2/10 G 5/15 had originally been selected in an attempt to isolate Nissl bodies (35,36,37). However, in the absence of any specific evidence for the presence of Nissl bodies in this fraction (or in any other) it was decided to leave this fraction unnamed, but rather to compare its chemical properties with those of other fractions. The greatest obstacle to the fractionation of Nissl bodies appears to be that chromatolysis may have destroyed the major portion of them (if not all) by the time such a fraction is obtained.

When compared to liver tissue brain tissue is structurally more diversified. This presents a difficulty in the study of the intracellular distribution of enzymes by differential centrifugation. The results of fractionation

* The conditions for the nuclear fraction are an exception. They are such that no nuclei are left in the cytoplasmic material, a minimum amount of the latter being sacrificed along with the nuclei.

TABLE XVI - Various conditions under which Mitochondria and Microsomes have been isolated.

Mitochondria	Microsomes	Medium	Tissue	Authors
0.6/30 V 2/10*	-	0.85% NaCl	Liver	Hoerr, 1943. (39)
1.5/30 G 2/25	18/4 G 18/90	"	"	Claude, 1946 (10)
0.7/10 G 5/10	5/10G 57/60	0.25M sucrose	"	Schneider & Hogeboom, 1950 (40)
1.6/10 G 29/10	29/10 G 130/30	0.88M "	"	" " " "
1.2/10 G 18/30	18/30 G 41/150	" "	"	Palade, 1951 (13)
0.6/10 G 8.5/10	8.5/10 G 20/30	0.25M "	" & kidney	Berthet et al,1952(14)
1.5/10 G 12/15	12/15 G 23/30	" "	Brain	Brody & Bain, 1952 (16)

* The numerators stand for rounds per minute in thousands, and V stands for angular velocity (standard size International Centrifuge).

studies therefore represent an average value for the "brain cell". This difficulty is not as great with the more homogeneous liver tissue, and this fact has to be born in mind when working with brain.

Distribution of the Phosphatases. The conditions of assay for the three phosphatases were such that the amount of inorganic phosphorus liberated bears a linear function with time for at least the first 70 minutes of incubation.

The acid phosphatase of liver has been shown by the following authors to occur mainly in what is called the mitochondrial fraction. Palade (13) using 0.88M sucrose obtained 40 percent of the activity in fraction 1.2/10 G 18/30. Berthet et al (14) using 0.25M sucrose needed less centrifugal force and obtained about 60 percent of the activity in fraction 0.6/10 G 8.5/10. The nuclear fraction with both workers contained very little activity which led them both to suggest that the nuclei may be devoid of this enzyme and that the little activity found in this fraction may be solely due to cytoplasmic contaminants. The acid phosphatase of dog cerebrum (tables I to IV) is found to be in general very much like that of liver. About half of the activity is found in the two fractions 1.2/10 G 5/15 and 5/15 G 20/30. (tables III and IV). These fractions together correspond roughly to what

others have called mitochondria. The acid phosphatase of cerebral tissue is also sensitive to mechanical damage as de Duve et al have reported for liver tissue (26,27). This is shown by the experiment described in Fig.3 and table X. In experiment II the activity of the two above mentioned fractions does not quite reach 50 percent, an unusually large amount being lost to the final supernatant possibly the result of excessive mechanical damage. The distributions of experiment I are not very representative of true conditions since an appreciable amount of cytoplasm contaminates the nuclear material.

The results of experiments II to IV on alkaline phosphatase seem to agree with what Richter and Mullin (15) have already reported on cerebral cortex, and Dounce (38) on liver tissue. There is a high concentration of this enzyme in the nuclei. These authors, however, did not study the distribution in the cytoplasm. This was studied by Novikoff et al (12) on liver tissue using phenyl phosphate as substrate. The distribution they find is altogether different from that of brain tissue.

The latter also find a completely different distribution when A5P is used as a substrate. Unlike brain tissue they find an equally high concentration of enzyme in the nuclear and mitochondrial fractions (40-45 percent).

It appears that the method presented above on the assay of 5-nucleotidase by denaturation with heat

deserves further study. Since this is a reaction of the first order the value of $t\frac{1}{2}$ called the half period of the reaction ought to be the same regardless of the initial concentration.* The specific reaction rate, k,* or the slope of the log. activity - time plot, being also characteristic, should be the same in all cases. From table XVII it appears that each fraction has a different half period (and slope). This can hardly mean that each fraction contains a different 5-nucleotidase, since the original homogenate also has a half period very far from the average of the other brain fractions. If the cause for such variations were due to an initial latent period (or period of activation) before the onset of denaturation then the half periods of the various fractions should not differ more than 30 minutes. The data in table XVII show that there is no correlation between the half period of a fraction and its protein (mg N per cc.) or inorganic phosphate concentration.

It is possible that these variations on the value of $t\frac{1}{2}$ are due to experimental error. It will be noticed from Fig. 6, that the curve (disregarding the hump) approaches the ordinate at a narrow angle. This makes extrapolation to zero time subject to more error than if the curve approached the ordinate almost perpendicularly. This error may be partly overcome by denaturating the enzyme at a lower temperature than 60°C.

---

* See equation page 26.

TABLE XVII - Half-period values in the destruction of 5-nucleotidase in the various fractions obtained in experiment III.

Fraction	Slope ($\log_{10}$ units/min or $k/2.30 \times 10^3$)	half period ($t_{\frac{1}{2}}$ in min.)	mg N per cc.	Inorganic phosphate µg/cc
G 1.2/10	-1.67	180	10.1	15.2
1.2/10 G 5/15	-2.60	115	15.9	24.0
5/15 G 20/30	-2.90	110	11.6	28.4
20/30 G 30/90	-1.90	160	1.9	4.3
S 30/90	-1.59	188	3.08	22.3
Original Homog.	-3.32	90	7.86	29.0

SUMMARY

1. Dog's cerebrum was homogenized in 0.25M sucrose by the Potter-Elvehjem technique and fractionated by differential centrifugation.
2. The following five consecutive fractions were obtained:
   (a) a "nuclear" fraction after 10 minutes at 1,200 G containing also erythrocytes and cytoplasmic material as impurities (G 1.2/10),
   (b) 1.2/10 G 5/15, cytoplasmic granules after 15 minutes at 5,000 G spheroidal in shape measuring from 1 to 4.5 micra,
   (c) 5/15 G 20/30, cytoplasmic granules after 30 minutes at 20,000 G measuring 1 micron or less in diameter,
   (d) 20/30 G 30/90, cytoplasmic granules after 90 minutes at 30,000 G, submicroscopic in size, and
   (e) S 30/90, the final supernatant fluid
3. The phosphatase distribution in these fractions is as follows:
   (a) Acid phosphatase activity is mainly located in the larger cytoplasmic particles (1.2/10 G5/15 and 5/15 G 20/30). Mechanical damage will easily release part of it into the surrounding fluid.
   (b) Alkaline phosphatase has the greatest concentration - in terms of specific activity - in the nuclear fraction and in a fraction consisting of submicroscopic particles (20/30 G 30/90).

(c) 5-Nucleotidase activity is more uniformly distributed throughout the cytoplasmic fractions than the other two enzymes. Its specific activity is greater the smaller the cytoplasmic particles of the fraction.

4. The soluble nitrogen (total nitrogen) in the final supernatant increases the greater the mechanical damage. The ~~cytoplasmic~~ cellular particles lose part of their soluble nitrogen on dilution with 0.25M sucrose.

5. A method of measuring the activity of 5-nucleotidase when contaminated with alkaline phosphatase is presented. It is based on the fact that alkaline phosphatase is rapidly destroyed at 60°C while 5-nucleotidase is more heat-stable. The destruction of the latter enzyme by heat is a reaction of the first order. The "true" activity of the enzyme is determined by plotting the logarithm of its activity against time of treatment at 60°C and extrapolating to zero time.

6. By this method it was found that the non-specific alkaline phosphatase exhibited very little activity under the conditions of assay for 5-nucleotidase and in the presence of this enzyme.

REFERENCES

An asterisk (*) after a reference denotes that an abstract only was available.

(1) K.V. GIRI & N.C. DATTA (1935). Current Sci. (India) 4:324 (*).

(2) K.V. GIRI & N.C. DATTA (1936). Biochem.J. 30: 1089-1096.

(3) F. CEDRANGOLO & A. RUFFO (1938). Arqh. Sci. Biol. Sperim. (Italy) 24:59-69.

(4) E. BAMANN & E. RIEDEL (1934). Z. Physiol. Chem. 229: 125-50.

(5) E. WALDSCHMIDT-LEITZ & W. NONNENBRUCH (1935) Naturwissenschaften 23: 164. (*).

(6) J. ROCHE (1931). Biochem. J. 25: 1724-33.

(7) J. REIS (1937). Enzymologia 2: 110-16.

(8) J. REIS (1937). Enzymologia 2: 183-190.

(9) R.R. BENSLEY & HOERR (1934). Anat. Rec. 60: 449-55.

(10) A. CLAUDE (1946). J. Exp. Med. 84: 51-59; 61-89.

(11) W.C. SCHNEIDER & G.H. HOGEBOOM (1951). Cancer Research 11: 1-22.

(12) A.B. NOVIKOFF, E. PODBER, & J. RYAN (1950) Fed. Proc. 9:210.

(13) G.E. PALADE (1951). Archiv. Biochem. 30: 144-58.

(14) J. BERTHET & C. de DUVE (1952). Biochem. J. 50: 174-81.

(15) D. RICHTER & R. P. HULLIN (1951). Biochem. J. 48: 406-10.

(16) T.M. BRODY & J.A. BAIN (1952). J. Biol. Chem. 195: 685-96.

(17) H. CHANTRENNE (1947). Biochim. Biophys. Acta 1: 437-48.

(18) V.R. POTTER & C.A. ELVEHJEM (1936). J. Biol. Chem. 114: 495-504.

(19) C.H. FISKE & Y. SUBBAROW (1925). J. Biol. Chem. 66: 375-400.

(20) W.N. UMBRETT & V.S. BOND (1936) Ind. and Eng. Chem., Anal. Ed. 8: 276.

(21) S.E. KERR (1940) J. Biol. Chem. 132:149.

(22) T.S. MA & G. ZUAZAGA (1942). Indust. and Eng. Chem. Anal. Ed. 14: 280-82.

(23) G.H. HOGEBOOM, W.C. SCHNEIDER, & G.E. PALADE (1948). J. Biol. Chem. 172: 619-35.

(24) E.V. COWDRY (1918). Carnegie Inst. Publication 271 : 39-160. *

(25) Van R. POTTER, R.O. RECKNAGEL, & R.B. HURLBERT (1951) Fed. Proc. 10: 646-53.

(26) C. de DUVE, J. BERTHET, L. BERTHET, & F. APPELMANS (1951) Nature 167: 389-90.

(27) J. BERTHET, L. BERTHET, F. APPELMANS & C. de DUVE (1951) Biochem. J. 50: 182-89.

(28) M.P. LAMBDEN (1950) Fed. Proc. 9:193.

(29) L.A. HEPPEL & R.J. HILMOE (1950) J. Biol. Chem. 188: 665-75.

(30) J.L. REIS (1951) Biochem. J. 48: 548-551.

(31) H.U. ZOLLINGER (1950). Experientia 6: 14-19. *

(32) A. CLAUDE & E.F. FULLAM (1945). J.Exp.Med. 81:51-62.

(33) R.L. SWANK & J. CAMMERMEYER (1949). J. Cell.Comp. Physiol. 34: 43-70.

(34) A.E. MIRSKY & H. RIS (1947). J.Gen. Physiol. 31:1-6.

(35) Sven INGVAR (1923). Archiv. Neurol. Psychiatry 10: 267-87.

(36) H.W. BEAMS & R.L. KING (1935) J. Comp.Neur. 61:175-89.

(37) A. ACRA & S.BOULOS (1951). Unpublished. Dept. of Biological Chemistry of the American University of Beirut; Beirut, Lebanon.

(38) A.L. DOUNCE (1950). Ann. N.Y. Acad. Sci. 50:982-99.

(39) N.L. HOERR (1943). Biol. Symp. 10: 185-231.

(40) W.C. SCHNEIDER & G.H. HOGEBOOM (1950). J. Biol. Chem. 183: 123-28.

.. ..

ADDENDUM

The experimental work presented in this thesis has raised several questions which remain unanswered. A continuation of these studies should, therefore, include investigation of the projects outlined below.

The Measurement of Enzyme Activity

Acid Phosphatase. It was found that the relative activity of acid phosphatase in the supernatant fraction S 30/90 increased upon dilution with water. A plot of enzyme concentration against activity gave the results shown in Fig. 14. Enzyme concentrations are expressed as fractions of the original undiluted sample, the latter being given the arbitrary concentration of 1.0. As shown by the graph, the relation between enzyme concentration and activity appears to be linear. When the straight line is extended to zero concentration there remains an apparent activity, M. (Fig. 14). If enzyme activity is represented as v, and enzyme concentration as E then, $v = kE + M$ where k and M are constants. This is not the case with crude 5-nucleotidase (containing alk.Pase) where the straight line appears to meet the origin (Fig. 15).

If there were neither activation nor inhibition on dilution then the relative activities of acid phosphatase ought to be the same at all enzyme concentrations. Thus a

plot of v/E against E should give a straight horizontal line as in the case of 5-nucleotidase (Fig. 16). However, with acid phosphatase the apparent activation upon dilution is more clearly seen in the plot of v/E against E (Fig.17).

This activation on dilution may be explained by one (or both) of the following hypotheses.

1. A natural inhibitor occuring together with the enzyme completes with substrate in forming a complex with the enzyme. Thus,

$$2E + I + S \rightleftharpoons \underset{\text{active}}{ES} + \underset{\text{inactive}}{EI} \quad \ldots\ldots\ldots (1)$$

where E = enzyme; I = inhibitor; and S = substrate Inhibition occurs because I removes part of E available for S. Now equation (1) represents essentially two reactions:

$$(ES) = k_e \ (E) \ (S) \quad \ldots\ldots\ldots\ldots\ldots (2)$$

and $$(EI) = k_i \ (E) \ (I) \quad \ldots\ldots\ldots\ldots\ldots (3)$$

where brackets are used to denote concentration, k_e and k_i being equilibrium constants. Upon dilution (E) and (I) decrease in the same proportion (equa.3) but (S) remains constant under the conditions of assay. Hence upon dilution the concentration of EI is decreased relatively more than that of ES. Since the activity of an enzyme is proportional to the enzyme-substrate complex, (ES), (41) then the above hypothesis may explain why acid phosphatase exhibits a relatively greater activity upon dilution.

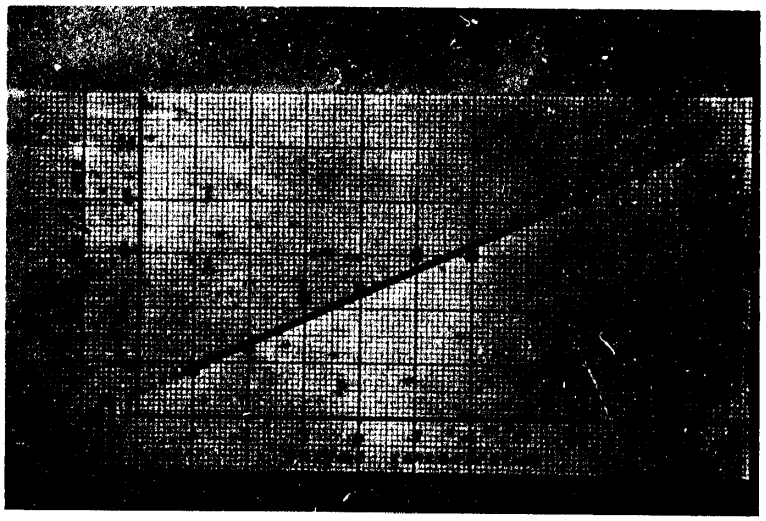

Fig. 14. The activity of acid phosphatase in fraction S 30/90 as related to enzyme concentration.

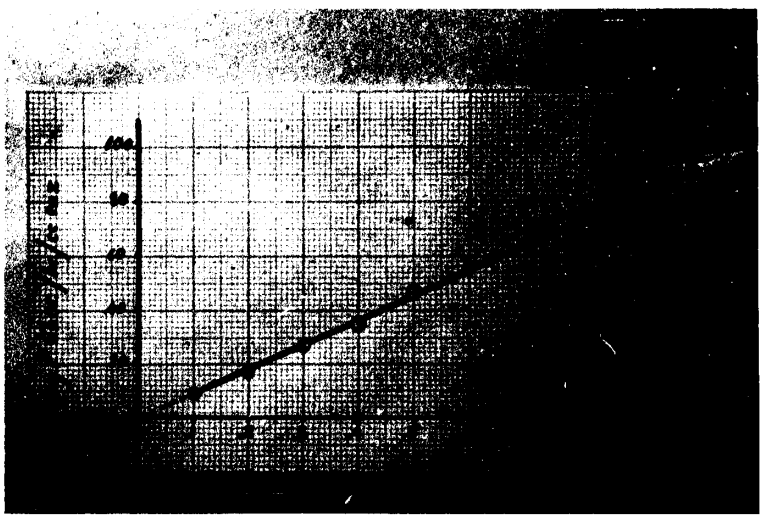

Fig. 15. The activity of "raw" 5-nucleotidase (containing also alkaline phosphatase) in fraction S 30/90 as related to enzyme concentration.

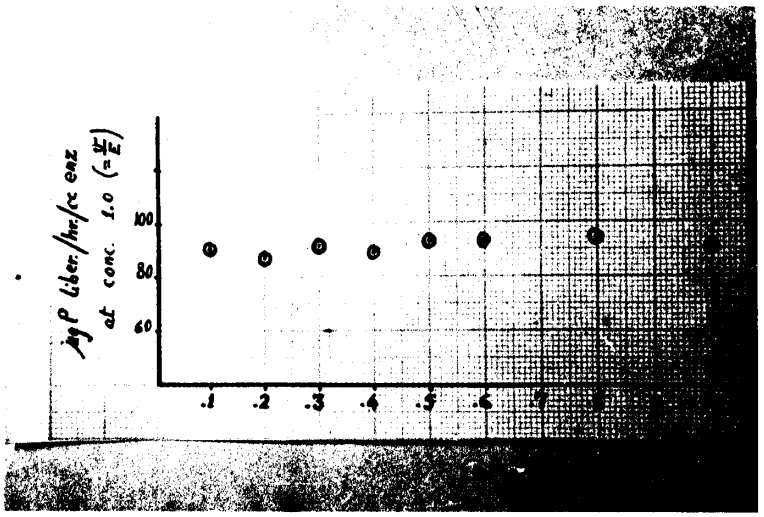

Fig. 16. Relative activity of 5-nucleotidase at different enzyme concentrations. This is plotted from the same data given in Fig. 15.

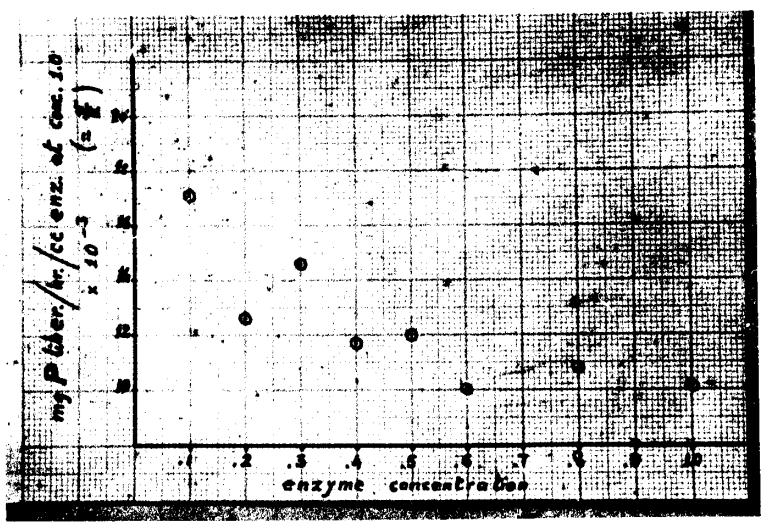

Fig. 17. Relative activity of acid phosphatase at different enzyme concentrations. This is plotted from the same data given in Fig. 14.

2. Part of the enzyme occurs in a bound inactive form probably linked to a protein. Dilution liberates the enzyme by denaturing the complex.

The second hypothesis can be tested in a procedure very similar to that followed with 5-nucleotidase. The destruction of the enzyme by heat can be observed at definite time intervals and the "true" activity can be calculated as was done with 5-nucleotidase.

Such a procedure, on the other hand, cannot be applied to test the first hypothesis unless the inhibitor, I, is more heat labile than the acid phosphatase. If this is not the case the measurement of the true activity of acid phosphatase presents a more complicated problem since the concentration of I is unknown.

It would be interesting to investigate whether each of the brain fractions behaves similarly, or whether the hypothetical acid phosphatase inhibitor is more concentrated in one particular fraction.

Heat Activation of Purified 5-Nucleotidase. It was shown that "raw" 5-nucleotidase was slightly activated by heating to 60°C (Fig. 6) for a short time. One explanation was that the enzyme is found partly in a bound inactive form (probably linked to a protein). Heat treatment released the bound enzyme. It would be interesting to find out whether purified 5-nucleotidase behaves similarly.

This enzyme can be purified by the technique of Heppel and Hilmoe with ammonium sulfate fractionation (29). If the enzyme is found partly linked to a protein then the purification procedure may either dissociate the protein or remove the protein-enzyme complex. The result would be an enzyme solution that is not activated by heat.

.. ..

The Intracellular Distribution of Enzymes

The Distribution of Pyrophosphate. An enzyme capable of hydrolyzing inorganic pyrophosphate was demonstrated in brain by Gordon (42,43). This enzyme — pyrophosphatase — had already been reported in muscle (44) and other tissues (45, 46). Its intracellular distribution was studied only in liver by Swanson (47). She finds the majority of the activity to be located in the soluble fraction of the cell.

As a pioneer experiment, therefore, the inorganic pyrophosphatase activity was determined in the brain fractions of experiment IV. The enzyme was assayed at pH 7.4 in the presence of 10^{-3}M tetrasodium pyrophosphate (Merck), 0.02M $MgCl_2$, and 0.03M veronal buffer. The rpocedure was the same as that described under Methods (page 9), except that the enzyme was incubated for only 20 minutes. The action of the Waring blendor was also tried on the enzyme, which was found to be very labile to mechanical damage (see table XIX). Activities are expressed as mg P liberated per hr. per cc. of enzyme.

The distribution of pyrophosphatase is similar to that of liver: the enzyme appears to be located mainly (70-80%) in the soluble fraction of the cell. This being the result of a single experiment, further confirmatory experiments are necessary.

TABLE XVIII - Inorganic pyrophosphatase distribution in experiment IV. The enzyme was assayed at pH 7.4 in the presence of 10^{-3} M tetrasodium pyrophosphate, 2×10^{-2}M $MgCl_2$, and 3×10^{-2}M veronal buffer. Activities are expressed as mg P liberated per hr. per cc. of enzyme.

Fraction	1st day after fractionation			4th day
	mg P liber/hr by fraction	percent of Homogenate	Specif. activ. ug P/hr/ mg N	mg P liber/hr by fraction
G 1.2/10	6.3	1.45	162	4.11
1.2/10 G 5/15	46.0	10.5	534	47.7
5/15 G 20/30	30.4	7.0	554	28.8
20/30 G 30/90	3.3	0.76	194	2.31
S 30/90	321.0	73.6	4700	267.6
Original Homog.	436.0	Total 93.0	159	449.0

TABLE XIX - The effect of the Waring blendor on brain pyrophosphatase. This was performed in the same experiment described in Fig. 3.

Fraction	mg P liber/hr per cc. enzyme	percent of E
E	1.12	100.0
W	0.561	50.0
EW	0.834	74.5
SW	0.270	24.1
SEW	0.399	35.6
SE	0.96	85.6

The Distribution of Ribonucleic Acid. Since 5-nucleotidase hydrolyzes ribonucleotides, the components of ribonucleic acid (RNA), any correlation between the distribution of nucleic acid and the enzyme might throw some light on the function of the latter.

The distribution of RNA was determined in a single experiment (No. IV) by the procedure of Schmidt and Thannhauser (48) modified for use with the Beckman spectrophotometer. After alkaline hydrolysis the ribonucleotides were determined spectrophotometrically at 257.5 mμ , the molar extinction coefficient per phosphorus being taken as 12070 - an average value (49). The results are shown in table XX. It appears that there is little correlation in the percentage distribution of the enzyme and that of RNA, yet both components are present in all the cerebral fractions. As a further study along this line the distribution of ribonuclease should be determined in the brain fractions.

The distribution of Phosphodiesterase. No phosphodiesterase has yet been reported in brain. This investigation would require the following experimental work:

a) a pH-activity curve,

b) a study of optimum substrate concentration,

c) a study of activators and inhibitors.

A suitable substrate for this enzyme is diphenyl phosphate.

TABLE XX - The distribution of ribonucleic acid in the cerebral fractions obtained in experiment IV. The total value as calculated from the original homogenate is 1.72 ug of RNA P per gr. fresh tissue.

Fraction	mg RNA P in fraction	percent of Homogenate		µg RNA P per mg total N
		RNA P	5-nucleotidase	
G1.2/10	0.575	19.9	10.3	14.8
1.2/10 G 5/15	0.640	22.1	16.9	7.4
5/15 G 20/30	0.518	17.9	30.6	9.4
20/30 G 30/90	0.241	8.3	19.6	14.2
S 30/90	0.70	24.2	18.0	10.2
Original Homog.	2.89	Total 92.5	To.95.4	10.6

References

(41) L. MICHAELIS & M.L. MENTEN. (1913). Biochim. Z. 49: 333-69. *

(42) J.J. GORDON (1949) Nature 164: 579-80.

(43) J.J. GORDON (1950). Biochem. J. 46: 96-99.

(44) LOHMANN (1928). Biochem. Z. 203: 172. *

(45) H.D. KAY (1928). Biochem. J. 22: 1446-48.

(46) B.NAGANNA & N.V.K. MENON (1947). Current Sci. (India) 16: 226. *

(47) M.A. SWANSON (1952). J. Biol. Chem. 194: 685-93.

(48) G. SCHMIDT & S.J. THANNHAUSER (1945). J. Biol. Chem. 161: 83-89.

(49) K.K. TSUBOI (1950). Biochim. Biophys. Acta 6: 202-209.

.. ..

I wish to express my gratitude to

Dr. Stanley E. Kerr for his help and

advice in the realization of this project.