PLACENTAL PHOSPHATASES

Z. AHMED* AND E. J. KING
Postgraduate Medical School of London (England)

(Received August 8th, 1958)

SUMMARY

1. Acid and alkaline phosphatase concentrations have been determined in human foetal blood, maternal blood and amniotic fluid. There appears to be no relation between the concentrations of the enzymes in the three fluids.

2. Acid and alkaline phosphatase, pyrophosphatase and 5-nucleotidase were determined in several human placentas and their corresponding amniotic fluids. The alkaline phosphatase activity of placental tissue was high. There appeared to be no constant relation between the amounts of the four different enzymes.

3. A difference between placental and erythrocyte pyrophosphatase was demonstrated. Their behaviour towards magnesium activation and inhibition by several cations was different.

4. Differential centrifugation of placental homogenates showed the acid phosphatase to be present mainly in the cytoplasm, alkaline phosphatase and pyrophosphatase mainly in the microsomes and cytoplasm, and most of the 5-nucleotidase in the microsomes, mitochondria and cytoplasm.

INTRODUCTION

The full-term human placenta is 6 to 8 in. in diam., and 1 in. thick, and weighs about 500 g. The uterine surface is made up of 15–20 convex areas which are called cotyledons. The foetal surface is not subdivided and is covered completely by the vascular mesoderm of the chorion with its large umbilical vessels. The placenta is the "transitory liver" of the foetus It is a storehouse of building and maintenance materials, and contains large amounts of carbohydrates, proteins, fats, vitamins, hormones, minerals and enzymes. The enzymes include alkaline and acid phosphatases, choline esterase, and proteolytic and glycolytic enzymes.

BUSSE[1] reported an active alkaline phosphatase in the placenta. BOTELLA LLUSIA[2] found it to increase in activity in the human placenta until the sixth or seventh month of pregnancy. NATAF[3] found that the phosphatase of pig's placenta increased as gestation proceeded. SEELICH AND GOMOLKA[4] found a similar ratio of alkaline to acid phosphatase in the placenta and amniotic fluid.

The plasma alkaline phosphatase was found by YOUNG, KING, WOOD AND WOOTTON[5] to rise during pregnancy. BERTHIOULT, BERGER AND GOUNET[6] likewise

* Present address: The General Company for Pharmaceuticals, S.A.E., 26 Sherif Pasha Str., Immobilia Building, Cairo.

References p. 324/325.

found a rise, followed by a post-partum fall. Similarly SPEERT, GRAFF AND GRAFF[7] found a three-fold increase by term and a rapid increase after delivery. The alkaline phosphatase of the cord blood was higher than that of maternal blood and there appeared to be no relation between the two. MARIANI, HEREDIA AND SENINO[8] likewise found an increase of plasma phosphatase during pregnancy but SERA[9] did not.

This paper reports a study of the occurrence and distribution of phosphate-splitting enzymes in the human placenta *i.e.* acid and alkaline phosphatases, pyrophosphatase and 5-nucleotidase. An account of the placental 5-nucleotidase, its behaviour and properties has already been published[10].

MATERIALS

Foetal and maternal blood. Simultaneous samples of the foetal blood from the cord and of maternal blood were collected. They were centrifuged and the plasma used for the determination of acid and alkaline phosphatase (see below).

Amniotic fluid. Enzyme activities were carried out on centrifuged-fresh amniotic fluid within 2 h of its collection.

Placental extract. The cord and membrane were removed, and the placenta washed free of blood with heparinized saline (0.9% salt solution), inserting canulae into the veins and arteries. A complete washing required about 5 l. The placenta was dried with blotting paper, minced in a mechanical mincer, weighed and an equal amount of water added. The mixture was homogenized in a Waring blender for 2 min, centrifuged and the supernatant separated for the enzyme estimations.

Placenta for histochemical examination. Specimens of the washed placenta were treated for histological phosphatases as described by PEARSE[11].

Placental homogenate for differential centrifugation. About 25 g of placenta were cut into small pieces with a knife. These were mixed with 80 ml ice-cold isotonic sucrose (0.25 *M*, adjusted to pH 7.2). The mixture was treated in a small Waring blender for 2 min, and diluted to 100 ml. This mixture was kept ice-cold. Part of it (10 ml representing 2.5 g) was further homogenized in a pre-cooled glass homogenizer, which was kept dipped in ice-cold water. The homogenizer tube was moved up and down to force the tissue against the grinding surface. Grinding was continued for 3 min. The procedure was considered satisfactory if there were very few cellular structures remaining.

The homogenizer was made from a 50-ml pyrex syringe from which the nipple had been cut and sealed off. The plunger was connected to a larger diameter rod. This was connected to a mechanical stirrer through a flexible joint of rubber tubing.

Erythrocyte haemolysate. The packed cells were washed twice with 0.9% salt solution, and then diluted with 9 vol. of distilled water, when a clear haemolysate was obtained. Enzyme determination were carried out immediately after preparing the haemolysate.

METHODS

Acid and alkaline phosphatases in blood plasma and amniotic fluid

The phenyl phosphate method of KING AND ARMSTRONG[12] was used as described by ABUL-FADL AND KING[13], the aminoantipyrine-ferricyanide procedure of KIND AND KING[14] being used to estimate the liberated phenol. Incubation times of 1 h for acid

References p. 324/325.

phosphatase pH 4.9 and 15 min for alkaline phosphatase pH 10 were used. Results are expressed as KING-ARMSTRONG units, *i.e.* mg phenol liberated in 1 h for the acid phosphatase, and in 15 min for the alkaline phosphatase.

Acid and alkaline phosphatase of placental extract

Two methods were used, *i.e.* the above, and the modification of the phenyl phosphate procedure (KING, ABUL-FADL AND WALKER[15]) in which the liberated phosphate is determined instead of the phenol. Acetate buffer is used in the latter procedure for acid phosphatase instead of the citrate usually employed for blood plasma. The two methods gave closely comparable results. KING-ARMSTRONG units are equal to $^1/_3$ mg of liberated P (phosphate) when free phosphate is estimated instead of free phenol (mol. wt. of phenol = 3 × at. wt. P).

Formol-stable acid phosphatase

Formaldehyde inactivates the acid phosphatase of the red cells, leaving that of some tissues, *e.g.* the prostate unaffected. The method is as above with the addition of 0.02 ml neutralized 40% formaldehyde solution.

Pyrophosphatase

Reagents: 0.01 *M* sodium pyrophosphate, 0.27 g in 100 ml distilled water, freshly prepared; pH 8.3 veronal buffer (for placental pyrophosphatase); pH 7.6 veronal buffer (for erythrocyte pyrophosphatase).

Procedure. 1 ml 0.01 *M* sodium pyrophosphate + 1 ml. H_2O (or 1 ml magnesium sulphate solution) + 4 ml buffer (pH 8.3 or 7.6) in a test tube are warmed in a water bath at 37° for 3 min. The enzyme solution (1 ml) is added and the tubes kept at 37° for 30 min. Samples of 1 ml are then pipetted into 15-ml flasks containing 1 ml 1 *N* H_2SO_4 + 5 ml. H_2O + 1 ml 5% ammonium molybdate + 0.5 ml 0.2% amino naphthol sulphonic acid, and water added to the mark and well mixed. A high concentration of strong acid must be avoided, in order to prevent hydrolysis of pyrophosphate. KUNITZ[16] found that 1 ml *N* H_2SO_4 in 15 ml was suitable. The colour should be read within 5 min. The pyrophosphatase unit is taken to be equal to 2 mg of orthophosphate P liberated in 60 min (2 ortho-phosphate = 1 pyro-).

5-Nucleotidase

Two centrifuge tubes containing 1.6 ml of 0.05 *M* veronal buffer solution of pH 7.5 and 0.2 of 0.01 *M* substrate solution also adjusted to pH 7.5 (sodium phenyl phosphate for the one and adenylic acid for the other) were placed in a water bath at 37° for about 5 min. Then 0.2-ml samples of enzyme solution (placental extract diluted ten times) were added and the tubes incubated for exactly 30 min. The enzyme reaction was stopped by 4 ml of 5% TCA solution. The samples were centrifuged and free phosphate estimated. Adenylic acid is hydrolysed by both the non-specific phosphatase and the 5-nucleotidase, phenyl phosphate only by the non-specific phosphatase; the difference between the two hydrolyses gives the 5-nucleotidase. The unit is equal to 1 mg liberated P per hour.

Enzyme activity at different pH

Phthalate buffer solution, (CLARK AND LUBS[17]) pH 2–6.6, and carbonate veronal

References p. 324/325.

buffer solutions (KING AND DELORY[18]) pH 7.5–10.5 were used. The exact pH of the mixed buffer and substrate solutions was measured with a Cambridge meter. In comparisons of enzyme activity at different pH value, a constant time of 30 min incubation was used with all substrates.

Differential centrifugation

The homogenate was centrifuged at 900 rev./min for 10 min. The supernatant was collected. The precipitate was washed with 1 ml 0.25 *M* sucrose at 0° and centrifuged for 10 min. This supernatant was added to the first. The residue containing the cell debris was kept in a refrigerator.

The combined supernatants were centrifuged at 1500 rev./min for 20 min, the new supernatant collected and the precipitate washed with 1 ml 0.25 *M* sucrose and centrifuged again for 10 min. The supernatants were combined. Some of the precipitate was fixed on a slide with 2 % formalin, stained with eosin-haematoxylin and examined microscopically. This was found to be mainly composed of nuclei. This constituted the nuclear fraction.

The combined supernatants were centrifuged in a superspeed centrifuge at 10,000 rev./min for 45 min, the supernatant collected, the precipitate washed with 0.5 ml of 0.25 *M* sucrose solution, and again centrifuged for 45 min. The supernatants were combined. The combined supernatants were now centrifuged at 24,000 rev./min for 90 min. A jelly-like precipitate appeared. This was the microsomal fraction. The supernatant was separated, and constituted the cytoplasmic fraction. All fractions were put immediately in the refrigerator after separation, and were examined for alkaline and acid phosphatase, pyrophosphatase and 5-nucleotidase activity without delay.

Histochemical alkaline phosphatase

The sample of tissue was fixed in cold formalin (4°, 8 h). Under these conditions artefacts are small and enzyme diffusion negligible. The method used depends on the hydrolysis of Na β-naphthyl phosphate and the instant reaction in situ of the liberated β-naphthol with diazotized α-naphthyl amine at pH 9.4. A red precipitate is produced at the site of phosphatase activity.

RESULTS

Phosphatases in blood plasma and amniotic fluid

Table I shows the results obtained for acid and alkaline phosphatase on maternal and foetal blood plasma and on amniotic fluid. The total cases examined were 15, but the maternal blood of one case and the foetal bloods of 9 were not available. All specimens of plasma and amniotic fluid were free of any traces of haemolysis. The maternal plasma acid phosphatases were all within the normal range of 0–4 units. The plasma alkaline phosphatases showed a slight but definite increase, one being twice the upper limit of normal (KING AND WOOTTON[19] give the normal range 3–12 units). Similar elevations of alkaline phosphatase in maternal blood have been noted by several authors referred to in the INTRODUCTION. Several explanations have been advanced for this increase during pregnancy. RAMSAY, THIERNS AND MAGEE[20] suggested that there is evidence of deficient calcium and phosphorous metabolism;

References p. 324/325.

TABLE I

ACID AND ALKALINE PHOSPHATASES IN MATERNAL AND FOETAL BLOOD PLASMA AND AMNIOTIC FLUID

(all expressed in units/100 ml)

No.	Maternal blood			Foetal blood			Amniotic fluid		
	Acid Total	F.S.*	Alkaline	Acid Total	F.S.*	Alkaline	Acid Total	F.S.*	Alkaline
1	4	4	12.5			7.9	4.5	3	7
2	1	1	15.3	5.2	1.5	14.1	4.7	3.0	2.3
3	2.2	0	17.2				1.6	1.1	1.1
4	1.1	0.1	11.3				3.3	2.2	1.1
5	2.6	2.6	6.8				2.6	2.1	1.1
6	2.6	2.6	10.0				1.0	0.5	3.7
7				32	3.5	0.3	48	5.3	9.6
8	1.6	0.4	10.8				2.4	1.9	2.5
9	38	3	0.3	7.7	7.7	13.8	29	2.6	6.6
10	4.4	1.6	10.5				3.8	2.5	4.2
11	4	3.5	21				1.1	0.6	1.4
12	1	0	14.5				2	0.5	4.5
13	0	0	10.3			0.8	0	0	18.5
14	2.1	2.1	25	4.2	4.2	16.6	4.7	1.8	8.3
15	1.7	0.9	14.2				1.7	1.2	1.5

* F.S. = formol-stable acid phosphatase.

BODANSKY[21] thought that occasional high values might be due to increased parathyroid stimulation.

Although the foetal bloods examined were very few, it seems that there is no definite relation between the alkaline phosphatase in maternal, foetal blood and amniotic fluid. Case No. 9, "ahydramnous" is remarkable for its very high acid phosphatase (though normal formol-stable acid phosphatase) in both maternal blood plasma and amniotic fluid, and an exceptionally low alkaline phosphatase in the maternal plasma. Case No. 7 also had very high acid phosphatases, this time in the foetal plasma, the maternal not having been done, and in the amniotic fluid. Several tissues of the body, particularly the red blood cells, have an acid phosphatase which is destroyed by formaldehyde: the large differences between the total and the formol-stable acid phosphatases in these two cases represent this formaldehyde-labile phosphatase. From what tissue it came is not known, but it could not have been from the blood cells since both plasmas and amniotic fluids were free of haemolysis. Case No. 7 was one of streptococcal toxaemia.

Phosphatases in placenta and amniotic fluid

The acid and alkaline phosphatases, pyrophosphatase and 5-nucleotidase were measured in 10 placentas and their corresponding amniotic fluids (Table II). The placental total acid phosphatase varied from 1.2 to 5 units whereas the formol-stable acid phosphatase was from 0 to 3 units; only a small proportion of the acid phosphatase of the placenta is therefore formaldehyde-labile like that of the red cells. The alkaline phosphatase varied from 8 to 41 units/g wet tissue; pyrophosphatase from 0.6 to 3.4 units and 5-nucleotidase from 0.4 to 10 units. There seems to be no obvious relation between any of the enzymes and the clinical conditions. DEMPSEY AND WISLOCKI[22] reported an increase in alkaline and acid phosphatase in pre-eclamptic placentas. The

References p. 324/325.

cases reported here are too few either to support or to contradict this finding. There is no relation between the placental enzymes and their corresponding values in the amniotic fluids. SEELICH AND GOMOLKA[4] claimed that the ratio between alkaline and acid phosphatase in placenta and amniotic fluid is constant. The figures in Table II do not support this. It is perhaps worth noting that the concentration of enzymes in the placentas (expressed as units/g wet tissue) are a hundred or more times those in the amniotic fluid (expressed as units/100 ml of fluid).

Histochemical phosphatase

The MENTEN, JUNGE AND GREEN[23] β-naphthol method as modified by GROGG AND PEARSE[24] gave excellent results (Fig. 1). Intense colour of the highly insoluble red dye allows the use of a very short incubation time. There was practically no staining of tissue nuclei, and the phosphatase activity was clearly limited to the placental epithelium. This result is similar to that described by DEMPSEY AND WISLOCKI and by DUMONT. Attempts to study acid phosphatase and 5-nucleotidase by histochemical means gave very weak staining reactions, although it appeared that their sites of principal activity were the same as that of alkaline phosphatase.

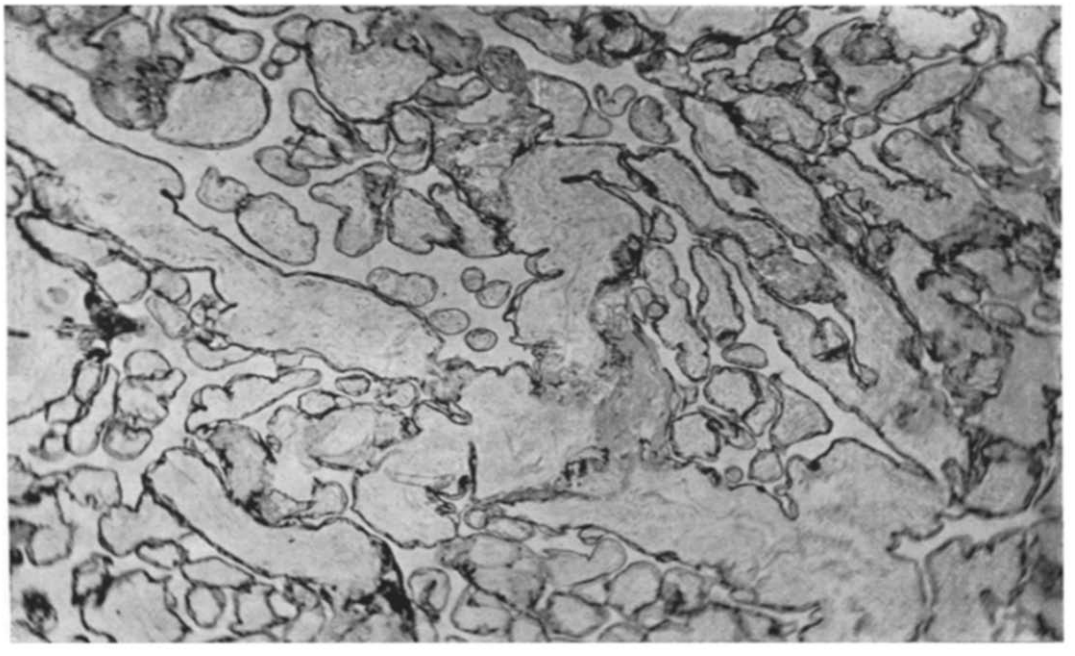

Fig. 1. Alkaline phosphatase in human placenta. Histological section stained by β-naphthol phosphate diazo method, × 100.

Some properties of placental phosphatases

Fig. 2 shows the results of hydrolysis of phenyl phosphate by fresh crude aqueous placental extract at different pH values. There are 2 peaks of activity, one at pH 5 and another between pH 10 and 10.3. The activity found at the alkaline pH is about 20 times greater than that at pH 5. Formol-stable enzyme was found to be 50 % of the total acid phosphatase. With added magnesium (final concentration 0.01 *M*) the acid

References p. 324/325.

TABLE II

PHOSPHATASES IN PLACENTAS AND THEIR CORRESPONDING AMNIOTIC FLUID

No.	Age of patient	Diagnosis	Placenta units/g wet tissue					Amniotic fluid units/100 ml				
			T.Ac.*	F.S**	Alk.***	Pyro.§	5-Nucleo.§§	T.Ac.*	F.S**	Alk.***	Pyro.§	5-Nucleo.§§
1	19	Normal labour, primigravida	2.2	1.8	41	3.4	10	3.1	2.2	6.25	1	—
2	30	Normal labour, multigravida (3rd)	1.2	1.2	16.6	0.6	2.6	4.7	1.5	4.5	0.8	1.8
3	31	Normal labour, multigravida (2nd)	2.5	1.2	20	1.2	3.2	4	4	2.5	0	0.5
4	32	Normal labour, primigravida	1.6	0	21.2	1.2	1.2	4.3	2.5	5.6	1.3	—
5	41	Normal labour, multigravida (3rd)	1.2	0.6	32	2.2	1.5	1.7	1.35	3.8	1.3	—
6	19	Hypertension, normal labour, primigravida	3.0	0	8	0.6	2.0	1.3	0	4.5	0.5	1.5
7	28	Hypertension, multigravida, (1st stillbirth, 2nd labour normal)	3	3	19	1.2	3.0	2.5	0	0	0.4	0.3
8	30	Mild hypertension, multigravida (2nd)	1.8	1.2	26	1.0	3.4	5	3.8	2.5	0.8	—
9	19	Pre-eclamptic toxaemia, primigravida	2.5	0	35	2.4	4.0	1.5	0.5	0	0	0.3
10	22	Pre-eclamptic toxaemia, primigravida	5	2.5	28	1.4	0.4	3	0	2.8	0.4	—
		Average	2.4	1.1	24.6	1.5	3.2	3.1	1.6	3.2	0.65	0.9

* T.Ac. = Total acid phosphatase.
** F.S. = Formol-stable acid phosphatase.
*** Alk. = Alkaline.
§ Pyro. = Pyrophosphatase.
§§ 5-Nucleo. = 5-Nucleotidase.

phosphatase was inhibited while the alkaline phosphatase displayed a 23 % increase of activity at the optimum pH.

Table III shows the effect of different concentrations of Mg, Co, and Zn on alkaline phosphatase of the crude placental extract, before and after dialysis against running tap water overnight and then against distilled water for a further 12 h. Mg was found to exhibit a maximum activation of the enzyme before dialysis at 0.005 *M*.

TABLE III

EFFECT OF DIVALENT CATIONS (Mg^{++}, Co^{++}, Zn^{++}) ON CRUDE PLACENTAL ALKALINE PHOSPHATASE

Molar concentrations		*mg P liberated/1 ml*	
		Before dialysis	*After dialysis*
No Cation added		72	63
1. Mg	0.02	90	78
	0.01	93	84
	0.005	99	75
	0.002	78	75
	0.001	76	74
	0.0005	75	72
	0.0002	72	70
	0.0001	72	69
2. Co	0.01	57	69
	0.005	81	78
	0.0025	93	76
	0.001	87	75
	0.0005	86	74
	0.0002	84	72
	0.0001	82	72
	0.00005	76	66
3. Zn	0.001	12	9
	0.0005	12	12
	0.0002	18	12
	0.0001	18	12
	0.00005	18	12
	0.000025	27	15
	0.00002	27	15
	0.00001	30	30

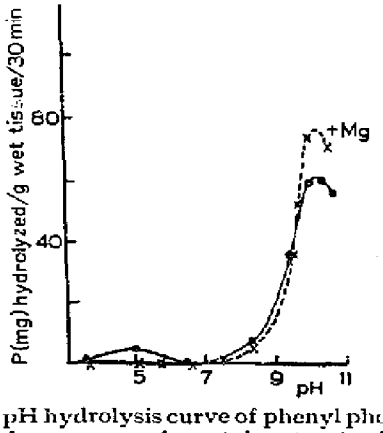

Fig. 2. pH hydrolysis curve of phenyl phosphate by fresh aqueous placental extract with and without added magnesium (0.01 *M* Mg).

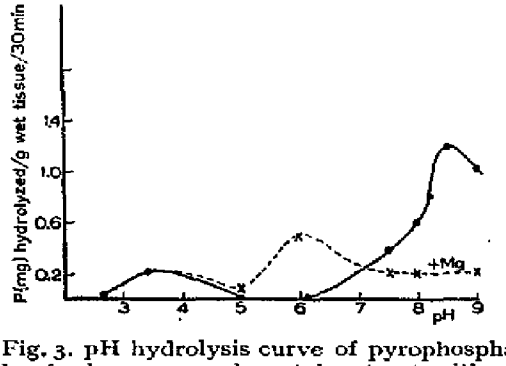

Fig. 3. pH hydrolysis curve of pyrophosphate by fresh aqueous placental extract with and without added magnesium (0.01 *M*).

References p. 324/325.

After dialysis, however, Mg was found to be maximally effective at 0.01 *M* showing that some Mg, which always accompanies alkaline phosphatase in crude extracts, was probably lost during dialysis. The diminution of enzyme activity after dialysis may be due to dilution by osmosis or partial denaturation or both. Co showed an inhibitory effect at 0.01 *M* but at lower concentrations activation of the enzyme occurred and was maximal at 0.0025 *M* and still evident at 0.00005 *M*. After dialysis activation was maximum at 0.005 *M*. Another experiment was performed in which both Co and Mg were added to the enzyme preparation in amounts required to produce maximum activation when used individually. In such an experiment the enzyme activity was found to be enhanced to a much higher level than that obtained by the use of either Mg or Co alone. Zn was inhibitory in all the dilutions used.

Fig. 3 shows the pyrophosphatase pH activity curve of crude placental extract with and without Mg (0.01 *M*). In the absence of Mg two peaks of activity appeared, one at pH 3.4 and the other at about pH 8.5. The latter activity is almost seven times as high as the former. With Mg, however, the two peaks of optimal activity were at 3.4 and at about 6.0. The component of very high activity at pH 8.5 without Mg is no longer apparent after the addition of Mg; instead a new one at pH 6 appeared. The activity at this new peak is not as high as that at pH 8.5. The activity at pH 6 with Mg is only 2.5 times as much as that at pH 3.4.

As with phosphomonoesterase, so pyrophosphatases also display isodynamic properties. Three are known, one with an optimum pH between 7.2 and 8.2 and activated by Mg. This is abundantly present in animal tissues (KAY[26], ROCHE AND BAUDOIN[27]). Another has an optimum pH between 5–5.5. This is not activated by Mg and is always associated with alkaline phosphatase in animal tissue and is abundant especially in liver (ROCHE AND BAUDOIN[27]). The third has an optimum activity between pH 3.2 and 4 (THOAI[28]).

Erythrocytes are amongst the tissues known to contain relatively high concentrations of pyrophosphatase (JENNER AND KAY[29]). It is optimally active at about 7.6 and is activated by Mg (NAGANNA AND MENON[30]). Experiments were carried out to compare erythrocytes and placental tissue with respect to pyrophosphatase activity in the presence and absence of Mg.

Fig. 4 shows the effect of different concentrations of Mg ion on the erythrocyte and placental pyrophosphatases at their optimum pH values. Mg is indispensable for the activity of red blood cell pyrophosphatase; no hydrolysis occurred in its absence. Maximum activation occurred in the presence of 0.001 *M* Mg. Such a concentration of Mg was strongly inhibiting to the placental enzyme. This was activated with relatively lower concentrations, *i.e.* 0.00025 and 0.000025 *M*.

NAGANNA AND MENON[30] found that without added Mg the erythrocyte pyrophosphatase activity was negligible and that 0.02 *M* increased the acivity more than 100-fold. KUNITZ[16] found that yeast pyrophosphatase has an optimum activity round pH 7 and that it is activated by 0.003 *M* Mg.

Zn, Co and Ca inhibited placental pyrophosphatase activity. Inhibition ceased at about 0.000025 *M* in the case of Zn and Co. Ca was the least inhibiting of the three and at 0.00001 *M*, Ca showed some activation. These results are shown in Table IV.

NAGANNA AND MENON[30] found Zn, Co and Ca to inhibit erythrocyte pyrophosphatase, but in concentrations different from those shown here for placental

References p. 324/325.

TABLE IV

ACTION OF ZINC, COBALT AND CALCIUM IONS OF DIFFERENT MOLAR CONCENTRATIONS ON PLACENTAL PYROPHOSPHATASE

No Cation added		Units/ml						
		0.002	0.001	0.0005	0.0002	0.0001	0.00001	0.000025
2.4	Zn^{++}	0.15	0.30	0.45	1.3	1.3	2.1	2.3
2.4	Co^{++}	0.15	0.30	0.67	1.5	2.1	2.3	2.4
2.4	Ca^{++}	0.22	1.6	1.7	2.0	2.0	2.8	2.4

pyrophosphatase. The two enzymes differ in their optimum pH, and their activation by Mg and in their inhibition by several metals.

The rates of hydrolysis of adenylic acid and phenyl phosphate by placental extract at varying pH values are represented in Fig. 5. The curves are similar to those described by REIS[31] for the tissues where a strong alkaline phosphatase and 5-nucleotidase are present together, *e.g.* human ossifying cartilage or choroid plexus. At about pH 9 the rate of hydrolysis of phenyl phosphate is much higher than that of adenylic acid; the reverse applies at pH 7.5. This fact may have some importance as concerns the physiological significance of phosphatases and indicates the procedure to be followed in measuring 5-nucleotidase activity.

The steep descent of the curve at low pH values suggests the occurrence of phosphorylation in this region.

The alkaline phosphatase and 5-nucleotidase activity in 16 placentas were estimated. The results are shown in Table V. The mean value of non-specific phosphatase is 1.3 mg P/g tissue/h when phenyl phosphate was used as substrate. Subtracting this from the mean value obtained when adenylic acid was used as substrate, one obtains an approximate value for 5-nucleotidase activity. The results obtained fall over a wide range. No correlation exists between those obtained for specific and non-specific phosphatase activity.

It was of interest to check how the methods used for alkaline phosphatase purification affected 5-nucleotidase activity. The purified alkaline phosphatase showed no 5-nucleotidase activity. We found that autolysis of placenta in the presence of

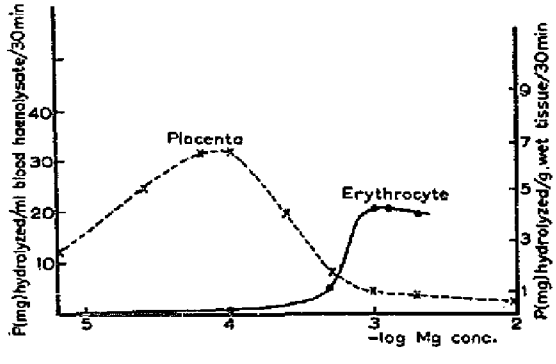

Fig. 4. Effect of different magnesium concentrations on placental (at pH 8.3) and erythrocyte (at pH 7.6) pyrophosphatases.

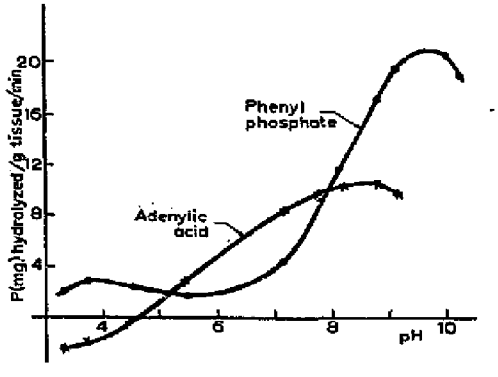

Fig. 5. pH hydrolysis curves on phenyl phosphate and adenylic acid by placental extract.

TABLE V

ALKALINE PHOSPHATASE (NON-SPECIFIC PHOSPHATASE) AND 5-NUCLEOTIDASE (SPECIFIC PHOSPHATASE) ACTIVITIES IN 16 HUMAN PLACENTAL EXTRACTS

Enzyme activity expressed as mg P liberated/g tissue/h at pH 7.5.

	Hydrolysis of:		*5-Nucleotidase*	*Ratio 5-nucleotidase/ non-specific phosphatase*
	Phenyl phosphate (non-specific phosphatase activity)	*Adenylic acid (non-sp. phosphatase + 5-nucleotidase)*		
Average:	1.3	3.9	2.6	2.0
Range:	0.5–3.0	2.0–5.5	0.4–5.1	0.3–5.2

toluene and ethyl acetate destroyed the 5-nucleotidase, while autolysis in the presence of a few drops of chloroform did not affect it. From this it is obvious that the 5-nucleotidase is destroyed during the first step of the procedure usually used for the alkaline phosphatase.

Differential centrifugation

Table VI shows the results of fractionating placental tissue, and for comparison gives those obtained by NOVIKOFF *et al.*[32]. More than half the acid phosphatase was found in the cytoplasm. The mitochondrial and microsomal fractions each contain 14% while there was none in the nuclei. The cell debris contained only 7%; this may be due to inefficient washing of the cell debris. The sum of the activities of the fractions was 92% of that measured in the whole tissue homogenate.

The alkaline phosphatase was present mainly in the cytoplasmic (37%) and microsomal fractions (33%) and mitochondria (13%). The remainder was found in the nuclear fraction (7%) and cell debris (8%). The high alkaline phosphatase activity

TABLE VI

DISTRIBUTION OF PHOSPHATASES IN PLACENTAL CELL FRACTIONS (UNITS/g TISSUE)

	Acid phosphatase		*Alkaline phosphatase*			*Pyrophosphatase*			*5-Nucleotidase*	
	Units	*%*	*Units*	*%*	*+ Mg Units*	*Units*	*%*	*+ Mg Units*	*Units*	*%*
Total	2.8	100	24	100	30.8	2.2	100	5.2	4.5	100
Residue (cell debris)	0.2	7	2.0	8	2.4	0.2	9	0.4	0.8	18
Nuclear fraction	0	0	1.6	7	1.7	0	0	0	0.1	2
Mitochondria	0.4	14	3.0	13	3.2	0.2	9	0.7	1.0	22
Microsomes	0.4	14	8.0	33	9.6	0.8	36	1.6	1.6	35
Cytoplasm	1.6	57	8.8	37	10.8	0.8	36	1.9	1.0	22
Recovery (%)		92		98			90			99
	Distribution in rat liver (NOVIKOFF *et al.*[32])									
Total		90–100		85–105						85–105
Nuclear fraction		5–10		10–18						40–50
Mitochondria		35–40		17–20						40–45
Microsomes		5–10		0–10						5–10
"Supernatant"		35–50		55–70						10–15

References p. 324/325.

of the cytoplasmic fraction may be due to the presence of smaller particles which could not be completely sedimented by the centrifugal force available.

The distribution of pyrophosphatase is more or less the same as that of alkaline phosphatase. One third of the 5-nucleotidase was in the microsomal fraction and one quarter in the mitochondria and cytoplasm. The large amount found in the cell debris could account for the low content of the nuclear fraction.

DOUNCE's[33] finding of a higher alkaline phosphatase in the nuclei was no doubt due to contamination by microsomal particles, for ALLFREY *et al.*[34], using a floatation technique for the isolation of nuclei, has shown that they are almost devoid of alkaline phosphatase activity.

It is not clear whether mitochondria do in fact have any appreciable alkaline phosphatase activity, and this would be simpler to ascertain if there were no doubt concerning the identity of the so-called "mitochondria". HIRS *et al.*[35] clearly recognised the possibility of small mitochondria which would sediment with the microsomal fraction. The results obtained by them using kidney of both rat and rabbit suggest that the mitochondria do in fact carry some phosphatase activity. However, it is clear that the microsomal fraction carries by far the greater activity.

DISCUSSION

The placenta is a very rich source of enzymes particularly of alkaline phosphatase. There seems little doubt that this high content of alkaline phosphatase is formed in the placental tissue itself. What its role is in the placenta is not clear, but it may take part in the exchange of various substances between mother and foetus as suggested by DUMONT. Its activity in the placenta is comparable with that in the intestinal mucosa and in the kidney, both tissues in which active transfers are taking place. The several phosphatase enzymes may also have a part in the metabolism of the placenta itself. It is hoped that further work on placental enzymes may yield information of both scientific and clinical value.

REFERENCES

1 O. BUSSE, *Z. Physiol. Chem. Hoppe Seyler's*, 242 (1936) 271.
2 J. BOTELLA LLUSIA, *Rev. clin. espan.*, 2 (1941) 334.
3 B. NATAF, *Compt. rend. soc. biol.*, 147 (1953) 1564.
4 F. SEELICH AND H. E. GOMOLKA, *Enzymologia*, 15 (1952) 96.
5 J. YOUNG, E. J. KING, E. WOOD AND I. D. P. WOOTTON, *J. Obstet. Gynaecol. Brit. Empire*, 103 (1946) 251.
6 R. BERTHIOULT, R. BERGER AND J. GOUNET, *Rev. Franc. gynecol.*, 47 (1948) 347.
7 H. SPEERT, S. GRAFF AND A. M. GRAFF, *Am. J. Obstet. Gynecol.*, 59 (1950) 148.
8 A. MARIANA, J. L. HEREDIA AND O. E. SENINO, *Rev. Fac. Cienc. med. Cordoba*, 9 (1951) 181.
9 G. C. SERA, *Acta Gynaecol. Scand.*, 1 (1950) 143.
10 Z. AHMED AND J. L. REIS, *Biochem. J.*, 69 (1958) 386.
11 A. G. E. PEARSE, *Histochemistry: theoretical and applied*, J. & A. Churchill London, 1953.
12 E. J. KING AND A. R. ARMSTRONG, *Can. Med. Assoc. J.*, 31 (1934) 376.
13 M. A. M. ABUL-FADL AND E. J. KING, *Biochem. J.*, 44 (1949) 435.
14 P. R. N. KIND AND E. J. KING, *J. Clin. Pathol.*, 7 (1954) 322.
15 E. J. KING, M. A. M. ABUL-FADL AND P. G. WALKER, *J. Clin. Pathol.*, 4 (1951) 85.
16 M. KUNITZ, *J. Gen. Physiol.*, 35 (1952) 423.
17 A. B. CLARK AND H. A. LUBS, *J. Biol. Chem.*, 25 (1916) 479.
18 E. J. KING AND G. E. DELORY, *Enzymologia*, 8 (1940) 278.
19 E. J. KING AND I. D. P. WOOTTON, *Micro-Analysis in Medical Biochemistry*, J. & A. Churchill, London, 1957.

[20] J. RAMSAY, V. T. THIERNS AND MAGEE, *Brit. Med. J.*, 1 (1938) 1199.
[21] M. BODANSKY, *J. Biol. Chem.*, 164 (1939) 321.
[22] E. W. DEMPSEY AND G. B. WISLOCKI, *Am. J. Anat.*, 80 (1947) 1.
[23] M. L. MENTEN, J. JUNGE AND M. H. GREEN, *J. Biol. Chem.*, 153 (1944) 471.
[24] E. GROGG AND A. G. E. PEARSE, *Nature*, 170 (1952) 578.
[25] M. DUMONT, *Presse méd.*, 23 (1937) 535.
[26] H. D. KAY, *Biochem. J.*, 22 (1928) 1446.
[27] J. ROCHE AND J. BAUDOIN, *Compt. Rend. Soc. Biol.*, 137 (1943) 245.
[28] N-V. THOAI, *Bull. Soc. chim. biol.*, 34 (1941) 264.
[29] H. D. JENNER AND H. D. KAY, *J. Biol. Chem.*, 93 (1931) 733.
[30] B. NAGANNA AND V. K. N. MENON, *J. Biol. Chem.*, 174 (1948) 501.
[31] J. L. REIS, *Biochem. J.*, 46 (1950) XXI.
[32] A. B. NOVIKOFF, E. PODBER AND J. RYAN, *Federation Proc.*, 9 (1950) 210.
[33] A. L. DOUNCE, *J. Biol. Chem.*, 147 (1943) 685.
[34] V. ALLFREY, H. STERN AND A. E. MIRSKY, *Nature*, 169 (1952) 128.
[35] C. H. W. HIRS, W. H. STEIN AND S. MOORE, *J. Am. Chem. Soc.*, 73 (1951) 1893.

EFFECT OF INHIBITORS ON LYTIC ENZYME SYNTHESIS BY *BACILLUS SUBTILIS* R

M. H. RICHMOND*
Department of Biochemistry, University of Cambridge (Great Britain)
(Received October 30th, 1958)

SUMMARY

8-aza-guanine has been shown to be a potent inhibitor of lytic enzyme synthesis in *B. subtilis* R. Its action is detectable within 5 min of addition to the system and is complete before incorporation of the analogue into the RNA fraction of the cells is appreciable. 8-aza-guanine was also found to be a potent inhibitor of general protein synthesis in the cell whether measured by the incorporation of L-[1-^{14}C]-leucine, L-[1-^{14}C]phenylalanine, DL-[1-^{14}C]alanine or [^{14}C]glycine. Cell wall synthesis, as measured by the incorporation of DL-[^{14}C]alanine into the egg-white lysozyme sensitive portion of heat-killed *B. subtilis* R, was not effected significantly by concentrations of the analogue causing inhibition of protein synthesis.

A method is suggested whereby the amino acid composition of small quantities of cell wall may be determined.

INTRODUCTION

It has been shown previously[1,2] that, during exponential growth in simple synthetic media, cultures of *Bacillus subtilis* R synthesise an extracellular lytic enzyme similar to egg-white lysozyme. As the extracellular lytic activity at any instant is a constant proportion of the total enzyme activity in the culture, the appearance of lytic activity in the growth medium may be used as a measure of enzyme synthesis. In the past a

* Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7, England.

References p. 340.