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Synthesis and secretion of alkaline phosphatase *in vitro* from first-trimester and term human placentas

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The synthesis and secretion of alkaline phosphatases *in vitro* by human placental tissue incubated in organ culture were studied. First-trimester placenta synthesizes and secretes two different alkaline phosphatase isoenzymes (heat-labile and heat-stable), whereas in term placenta nearly all the alkaline phosphatase synthesized and secreted is heat-stable. The specific activities of alkaline phosphatases in first-trimester and term placental tissue remain constant throughout the time course of incubation. In the media, specific activities increase with time. Hence, alkaline phosphatase synthesis seems to be the driving force for its own secretion. The rates of synthesis *de novo* and of alkaline phosphatases were measured. The specific radioactivities of the secreted alkaline phosphatases were higher than the corresponding specific radioactivities in the tissue throughout the entire incubation period. The intracellular distribution of the alkaline phosphatase isoenzymes was compared.

Human tissue-specific alkaline phosphatase isoenzymes [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are frequently used as genetic markers in cell biology and as parameters of tissue or organ disease in medicine. Three different isoenzymes have been characterized; structural properties of the enzymes, as well as immunological, chemical inhibition and genetic studies, suggest the involvement of at least three structural genes (Mulivor *et al.*, 1978; McKenna *et al.*, 1979). The isoenzymes were accordingly classified as: (a) placental-type, (b) intestinal-type and (c) liver-, kidney- and bone-type alkaline phosphatases.

The human placental-type alkaline phosphatase is tissue-specific (Boyer, 1961; Sussman *et al.*, 1968; Fishman, 1974; Badger & Sussman, 1976) and is found in many non-trophoblastic tumours (Stolbach *et al.*, 1969; Fishman, 1969; Singer & Fishman, 1974; Luduena & Sussman, 1976). It appears that two major forms of alkaline phosphatase are characteristic of human placenta. In early pregnancy, the heat-stable (placental-type) alkaline phosphatase activity is low and increases progressively as gestation proceeds (Jeacock *et al.*, 1963; Curzen & Morris, 1968; Sussman & Bowman, 1968). The other form of alkaline phosphatase, which is heat-labile and indistinguishable from liver alkaline phosphatase by several criteria (including apparent molecular weight and immunological reactivity; Sakiyama *et al.*, 1979), appears specific for first-trimester placenta (Fishman *et al.*, 1976). Although this heat-labile form accounts for a major fraction of the total alkaline phosphatase early in gestation (Speeg *et al.*, 1977), its relative contribution declines as the heat-stable alkaline phosphatase activity increases with time. The alkaline phosphatase from term placenta (heat-stable) has been extensively studied (Sussman *et al.*, 1968; Harkness, 1968; Sussman & Gottlieb, 1969; Greene & Sussman, 1973; Doellgast *et al.*, 1977); the heat-labile isoenzyme from first-trimester placenta has also been characterized (Sakiyama *et al.*, 1979).

The placental-type human alkaline phosphatase, which often occurs in human non-trophoblastic tumours, has been considered a marker for malignant transformation (Rosen *et al.*, 1975). Yet the appearance of this heat-stable enzyme is not universal in human tumours, many of which produce heat-labile alkaline phosphatase (Sasaki & Fishman, 1973). Since the identification of two distinct forms

Abbreviation used: SDS, sodium dodecyl sulphate.

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of placental alkaline phosphatase, it has been suggested (Sakiyama *et al.*, 1979) that derepression of one of these two forms always follows malignant transformation.

Although the human placental alkaline phosphatases have been purified and well characterized, little has been reported on the mode of synthesis and secretion of these enzymes in placenta. To extend our knowledge of the alkaline phosphatases, we have chosen to investigate the mode and kinetics of synthesis *in vitro* and secretion of human placental alkaline phosphatases.

Experimental

Materials

L-[4,5-^{3}H]Leucine (sp. radioactivity 30.2 Ci/mmol) and [^{32}P]phosphate (carrier-free) were purchased from Kamag, Negev, Israel. *p*-Nitrophenyl phosphate (crystalline) and human placental alkaline phosphatase were obtained from Sigma. Incubation medium M199 was purchased from Bio-Lab, Jerusalem, Israel. Bovine serum albumin (Fraction V) was purchased from Armour Pharmaceutical Co., Chicago, IL, U.S.A. First-trimester placental tissue was obtained from therapeutic abortions. Term placental tissue was obtained from normal deliveries or Caesarian sections. Tissue was transported in cold 0.9% (w/v) NaCl solution containing streptomycin, penicillin and mycostatin, and was processed within 30 min.

Organ culture

Tissue was cut into pieces of 50–100 mg wet wt. each, washed in M199 medium and incubated in Petri dishes (3 cm diameter) containing medium supplemented with 0.75% (w/v) bovine serum albumin and 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 8.0). Incubation was carried out for 120 h, with replacement of radioactive medium every 24 h. Samples of tissue were washed twice and homogenized in 0.25 M-sucrose in TKMNE buffer [50 mM-Tris/HCl (pH 7.4)/25 mM-KCl/10 mM-$MgCl_2$/100 mM-NH_4Cl/0.5 mM-EDTA]. Homogenates were spun at 1000 *g* for 10 min; supernatant ('tissue') fractions and media were collected for analysis.

Protein and radioactivity determinations

Protein was measured as described by Lowry *et al.* (1951). The radioactivity of the hot 5% (w/v)-trichloroacetic acid-insoluble material was determined as described by Bollum (1965).

Subfractionation

Subcellular fractions of rough membrane, smooth membrane, free polyribosomes and rough membrane stripped of its ribosomes were isolated from homogenates as described by Gal *et al.* (1977). Bound polyribosomes were prepared as follows: the rough-membrane suspension was adjusted to a final concentration of 1.0% (w/v) sodium deoxycholate. This suspension was layered on a cushion consisting of 1.45 M-sucrose in TKMNE buffer and spun for 3 h at 78000 *g*. The bound-polyribosome pellet was suspended in 0.25 M-sucrose in TKM buffer [50 mM-Tris/HCl (pH 7.4)/25 mM-KCl/10 mM-$MgCl_2$].

Enzyme assay

Placental alkaline phosphatase was assayed as described by Bessey *et al.* (1946) with the following modifications: enzyme activity was assayed at 37°C and at pH 10.5 with *p*-nitrophenyl phosphate as the substrate, and the release of *p*-nitrophenol was measured as the A_{410}. By definition, 1 unit of enzyme releases 1 μmol of *p*-nitrophenol/min. Heat-stable alkaline phosphatase is that activity measured after preincubation of the fraction for 15 min at 65°C. Heat-labile enzyme activity is the difference between the activity with and without preincubation for 15 min at 65°C.

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-slab-gel electrophoresis was performed on 7.5% (w/v) acrylamide gels (Laemmli, 1970). To locate the alkaline phosphatase after electrophoresis, gels were incubated in substrate solution [0.5 mM-*p*-nitrophenyl phosphate/0.05 M-ethanolamine buffer (pH 10.6)/0.5 mM-$MgCl_2$] for 10–15 min at 25°C. The coloured bands were cut and placed in 0.5 ml of solution containing 24% (v/v) H_2O_2 and 2% SDS (1:1, v/v). Gel slices were dissolved by heating at 75°C for 2 h, and radioactivity was determined.

Preparation of ^{32}P-labelled alkaline phosphatase

^{32}P-labelled enzyme was prepared as described by Engstrom (1961), as modified by Milstein (1964).

Results

Synthesis and secretion of alkaline phosphatases by placental tissue in organ culture

The synthesis and secretion of alkaline phosphatases from first-trimester placental tissue incubated in organ culture is shown in Fig. 1. The specific activity of total alkaline phosphatase in tissue remained constant over the time course of incubation (120 h). In order to measure secretion of the enzyme into media, the enzyme activity was measured at 24 h intervals ('delta values'), and also calculated from these delta values as accumulation over the 120 h incubation period ('additive values'). Although the additive values of total alkaline phosphatase specific activity increased with time, the

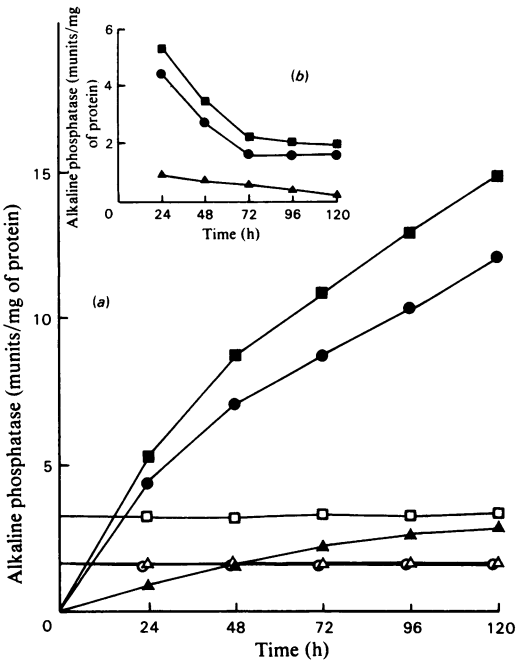

Fig. 1. *Specific activities of alkaline phosphatases from first-trimester placentas in organ culture*

Organ culture, alkaline phosphatase assays and protein determinations were performed as described in the Experimental section. The specific activities (munits of enzyme activity/mg of protein in secreting tissue) of alkaline phosphatases in tissue and media were measured. In Fig. 1(*a*) the specific activities shown in the media are additive with time, whereas those in Fig. 1(*b*) are delta values. Tissue: □, total alkaline phosphatase; △, heat-stable alkaline phosphatase; ○, heat-labile alkaline phosphatase. Medium: ■, total alkaline phosphatase; ▲, heat-stable alkaline phosphatase; ●, heat-labile alkaline phosphatase.

delta values for first-trimester placental incubation media decreased markedly (about 3-fold).

Heat-stable and heat-labile alkaline phosphatase activities could be found in first-trimester placental tissue and incubation media. As illustrated in Fig. 1, the specific activities of each isoenzyme in the tissue remained constant over time, with each contributing about 50% of the total alkaline phosphatase activity. As with the specific activities of total alkaline phosphatase, those of the heat-stable and heat-labile alkaline phosphatases in first-trimester-placental incubation media increased, if recorded as additive values over time (see Fig. 1*a*), whereas the delta values of each showed a decrease with time (see Fig. 1*b*). Hence the secretion of both the heat-stable and heat-labile alkaline phosphatases declines with time. However, although the rate of secretion of heat-stable alkaline phosphatase decreased linearly over 120h to 20% of the value at 24h, the rate of secretion of heat-labile alkaline phosphatase decreased much more rapidly over the first 72h, and then remained constant over the subsequent 48h of incubation (see Fig. 1*b*). These results suggest a different capacity of the first-trimester human placental tissue for secretion of each of the two alkaline phosphatase isoenzymes. Although the heat-labile fraction comprises about 50% of the specific activity from total alkaline phosphatase measured in the tissue of first-trimester placenta, this form accounts for about 80% of the total alkaline phosphatase activity in the media (this percentage remains constant over the course of incubation).

As with first-trimester placental tissue, the specific activity of total alkaline phosphatase in term placental tissue remained constant over the time course of incubation (Fig. 2). Nevertheless, the specific activities were about 5 times higher in term placenta than in first-trimester tissue (Figs. 1 and 2). The additive values for specific activity of total alkaline phosphatase in incubation media from term placenta increase (Fig. 2*a*), whereas the delta values showed a decrease with time (Fig. 2*b*). Although the first-trimester and term placental tissues each showed a decrease in capacity for secretion of alkaline phosphatases with time, there was a major difference in the pattern of this decline (Figs. 1*b* and 2*b*).

In term placental tissue the heat-stable alkaline phosphatase accounted for most of the measured total alkaline phosphatase specific activity (90–95% in the tissue, and more than 99% in the medium).

Several questions arose in connection with the decline in secretion of alkaline phosphatases with time. It was suggested that the activity of proteinases might increase in placental tissue with incubation time and that these enzymes might decrease the amount and/or activity of the alkaline phosphatases within the tissue as well as those secreted into the medium. In control experiments, which strongly negated this proposal, alkaline phosphatases were assayed in corresponding samples of homogenate and medium (for both first-trimester and term placentas); these corresponding homogenates and media were then recombined and incubated at 37°C for 4h. Full recovery of alkaline phosphatase activity resulted in the mixtures, i.e. no proteolytic breakdown of alkaline phosphatase could be detected in these experiments. Another relevant question was the stability of the alkaline phosphatases in the media under incubation conditions. A second set of control experiments was run, in which media were kept under incubation after the corresponding tissue had been removed and placed into fresh medium. There was no difference between the specific activity of the alkaline phosphatases measured in incubation

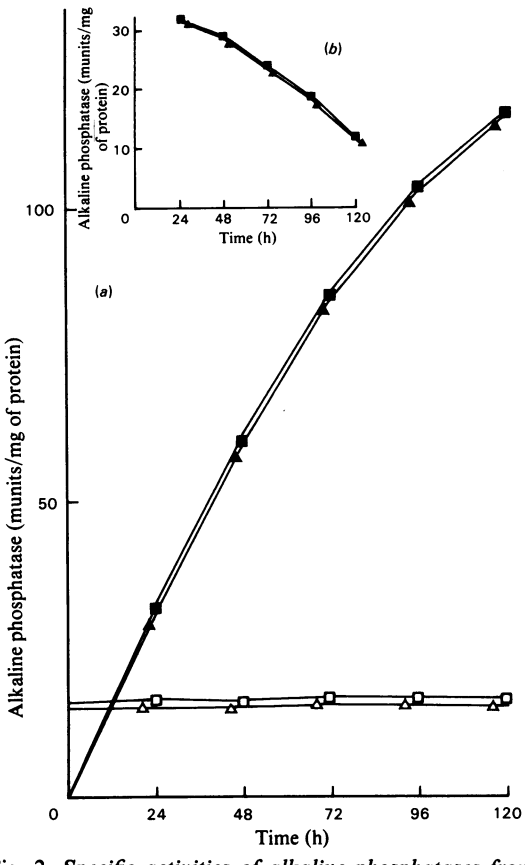

Fig. 2. *Specific activities of alkaline phosphatases from term placentas in organ culture*

Organ culture, alkaline phosphatase assays and protein determinations were performed as described in the Experimental section. The specific activities (munits of enzyme activity/mg of protein in secreting tissue) of alkaline phosphatases in tissue were measured. In Fig. 2(*a*) the specific activities shown in the media are additive with time, whereas those shown in Fig. 2(*b*) are delta values. Tissue: □, total alkaline phosphatase; △, heat-stable alkaline phosphatase. Medium: ■, total alkaline phosphatase; ▲, heat-stable alkaline phosphatase.

media frozen immediately after removal of tissue (until time of analysis) and those corresponding media exposed to additional incubation of up to 96h at 37°C. It therefore seems very likely that the alkaline phosphatases are stable in both tissue and media under organ-culture incubation.

The following control experiment virtually excluded the possibility of alkaline phosphatases in tissue, which were undetectable in our assay system owing to their membrane-bound or intravesicular location. Hence alkaline phosphatase assays were made on corresponding samples of homogenates, one of which was treated with deoxycholate (final concentration 1.0%) to dissolve its membranous components; identical specific activities were found for both untreated and deoxycholate-treated homogenates in each case (H. Galski, S. E. Fridovich, D. Weinstein, N. De Groot, S. Segal, R. Folman & A. A. Hochberg, unpublished work). Experiments in this section were repeated 25 times with a variation of ±5%; values for one representative experiment are given here.

Distribution of alkaline phosphatases among subfractions of first-trimester and term placental tissue

We were interested in the intermediate steps between synthesis and secretion of the alkaline phosphatases, and therefore subjected the postmitochondrial supernatant to further fractionation. To minimize tissue damage, homogenization was performed by using a glass homogenizer with a loose-fitting Teflon pestle at moderate speed. After subfractionation (see the Experimental section), each fraction was analysed according to the following criteria: amino acid-incorporation capacity, RNA/protein ratio, buoyant density and electron microscopy (Gal *et al.*, 1977). Despite gentle homogenization, there is always danger of enzyme redistribution. Control experiments were conducted in which ^{32}P-labelled alkaline phosphatase (for preparation, see the Experimental section) was added to the homogenization medium of fresh placental tissue. Homogenization and subfractionation were then performed, and each subfraction was analysed for radioactivity. About 94% of the radioactive enzyme was found in the postmicrosomal supernatant, and only small amounts were identified in the remaining subfractions (rough membrane, smooth membrane and free polyribosomes showed 3%, 1.5% and 1.5% respectively). These results suggest that very little, if any, of the soluble alkaline phosphatase is adsorbed to membranous fractions during homogenization and subfractionation.

A second set of control experiments was run. After preparation of subfractions, the specific activity of total alkaline phosphatase in each was determined. Each subfraction was then individually re-spun (gradient centrifugation) and re-precipitated twice, with total alkaline phosphatase specific activity being measured each time. These specific activities remained constant over all three measurements for a given fraction.

The findings from subfractionation of first-trimester human placenta are summarized in Table 1. As shown in Table 1(*a*), specific-activity values for heat-stable and heat-labile alkaline phosphatases exhibited very different patterns among the subfractions. When each isoenzyme activity was calculated as a percentage of that isoenzyme present in all

Table 1. *Specific activities and distribution of alkaline phosphatases among subfractions of first-trimester placenta*
Subfractionation, alkaline phosphatase assays and protein determinations were performed as described in the Experimental section. Specific activities for alkaline phosphatases in a given subfraction were calculated as munits of enzyme activity/mg of protein in that subfraction. Percentages appearing under 'within tissue' for each alkaline phosphatase represent the amount of enzyme (total munits) present in a given subfraction compared with the amount of that alkaline phosphatase found in all subfractions combined. In contrast, percentages appearing under 'within subfraction' for the heat-labile and heat-stable alkaline phosphatases represent the relative amounts of each isoenzyme within a given subfraction. In Table 1(*a*) the subfractions include postmicrosomal supernatant, rough membranes, smooth membranes and free polyribosomes. In Table 1(*b*) the postmicrosomal-supernatant subfraction was centrifuged, as described in the Results section. A pellet and three resultant supernatant fractions (Sup. 1, Sup. 2 and Sup. 3, collected from top to bottom respectively) were collected and analysed.

(*a*)

		Specific activity (munits/mg)			Isoenzyme distribution (% within tissue)			Isoenzyme distribution (% within subfraction)	
Subfraction	Alkaline phosphatase ...	Total	Heat-stable	Heat-labile	Total	Heat-stable	Heat-labile	Heat-stable	Heat-labile
Postmicrosomal supernatant		1.15	0.52	0.63	60.6	72.6	53.3	45	55
Rough membranes		4.22	1.08	3.14	18.0	12.0	21.6	25	75
Smooth membranes		3.69	1.10	2.59	19.1	15.0	21.7	30	70
Free polyribosomes		6.60	0.41	6.22	2.3	0.4	6.0	6	94

(*b*)

		Specific activity (munits/mg)			Isoenzyme distribution (% within postmitochondrial supernatant)			Isoenzyme distribution (% within subfraction)	
Postmicrosomal-supernatant subfraction	Alkaline phosphatase ...	Total	Heat-stable	Heat-labile	Total	Heat-stable	Heat-labile	Heat-stable	Heat-labile
Sup. 1		1.42	0.00	1.42	6.4	0.0	10.3	0	100
Sup. 2		0.96	0.00	0.96	8.9	0.0	14.2	0	100
Sup. 3		1.09	0.54	0.55	72.4	95.0	58.8	50	50
Pellet		1.33	0.21	1.12	12.3	5.0	16.7	16	84

fractions combined, the soluble subfraction (postmicrosomal supernatant) contained the largest percentage of the total alkaline phosphatase and also of both the heat-stable and heat-labile isoenzymes. The free polyribosomes contained the smallest percentages of total alkaline phosphatase and of both isoenzymes. As shown in Table 1(*a*), although the postmicrosomal supernatant contained roughly equal amounts of the heat-stable and heat-labile isoenzymes, the ratio of heat-labile to heat-stable alkaline phosphatases was about 3:1 in both the rough- and smooth-membrane subfractions, and 15:1 in the free polyribosomes.

Because the postmicrosomal supernatant contained 61% of the total alkaline phosphatase, this subfraction was centrifuged for 12h at 78000*g*. A pellet was obtained, and in the supernatant three distinct fractions were observed according to colour and turbidity. These were collected and analysed for alkaline phosphatases (see Table 1*b*). When each isoenzyme activity was expressed as a percentage of the isoenzyme present in all fractions combined, a completely different distribution was found between the heat-stable and heat-labile alkaline phosphatases among the fractions. Although the heat-stable alkaline phosphatase could not be found in fractions 1 and 2 (which were therefore combined as the upper supernatant), 95% of this isoenzyme was located in fraction 3 (the lower supernatant), with the remaining 5% in the pellet (see Table 1*b*). More than 50% of the heat-labile alkaline phosphatase was also present in the lower supernatant. In the pellet, the percentage of this heat-labile isoenzyme was about three times higher than that of the heat-stable alkaline phosphatase. Calculation of the percentage of each isoenzyme within a given fraction revealed that, although the lower supernatant preserved the approximate 1:1 ratio of heat-labile to heat-stable isoenzymes found in the postmicrosomal supernatant, the pellet showed a corresponding ratio of about 5:1.

The rough membrane stripped of its ribosomes and bound-polyribosome subfractions were prepared (see the Experimental section) from the rough-membrane subfraction. The stripped rough membrane and polyribosomes contained nearly equal amounts of total alkaline phosphatase, with specific activities of 5.8 and 2.6 munits/mg of protein

respectively. The bound-polyribosome subfraction therefore accounts for roughly 8% of the total alkaline phosphatase activity of the postmitochondrial supernatant. Although each fraction contained both the heat-labile and heat-stable isoenzymes, the heat-labile alkaline phosphatase was the predominant isoenzyme in the bound polyribosomes.

The findings from subfractionation of term placenta are summarized in Table 2. As shown in Table 2(*a*), specific-activity values for total alkaline phosphatase were highest in the smooth- and rough-membrane subfractions. No heat-labile alkaline phosphatase could be found in the postmicrosomal supernatant, smooth- or rough-membrane subfractions. The soluble fraction (postmicrosomal supernatant) contained the largest percentage of the alkaline phosphatase, whereas the free polyribosomes showed a negligible percentage of total alkaline phosphatase (similar results were found above for first-trimester placenta).

The postmicrosomal supernatant from term-placental subfractionation was centrifuged for 12h at 78000*g*; the resultant supernatant and pellet were collected and analysed for alkaline phosphatases (see Table 2*b*). The pellet contained only about 28% of the heat-stable alkaline phosphatase in both fractions combined. However, the specific activity of this isoenzyme was more than 10 times that of the supernatant.

The rough membrane stripped of ribosomes and the bound polyribosomes were prepared (see the Experimental section) from the rough-membrane subfraction. The stripped rough membrane contained about 90% of the total alkaline phosphatase activity of the two prepared subfractions, with the remaining 10% on the bound polyribosomes; the specific activities of this total alkaline phosphatase on stripped rough membrane and bound polyribosomes were 560 and 125 munits/mg of protein respectively. The bound-polyribosome subfraction thus accounts for roughly 2% of the total alkaline phosphatase activity of the original postmitochondrial supernatant, whereas the stripped rough membranes contained only the heat-stable alkaline phosphatase, although both isoenzymes were found on the bound polyribosomes.

Synthesis of alkaline phosphatase de novo

In order to study and to compare the synthesis *de novo* and secretion of alkaline phosphatases and of proteins by the placenta, first-trimester and term placental tissue were incubated in organ culture (see the Experimental section) containing [^{3}H]leucine as radioactive substrate in the medium. Media and tissue were then analysed to determine specific radioactivities of total protein and total alkaline phosphatase (see the Experimental section); results of these analyses are shown in Figs. 3 and 4. In first-trimester and term placental organ-culture systems there is an increase in radioactive incorporation into total alkaline phosphatase with time for both tissue and media (Figs. 3*a* and 4*a*).

Table 2. *Specific activities and distribution of alkaline phosphatases among subfractions of term placentas*
For details of methods and expression of results, see Table 1. In Table 2(*a*) the subfractions include postmicrosomal supernatant, rough membranes, smooth membranes and free polyribosomes. In Table 2(*b*) the postmicrosomal-supernatant subfraction was centrifuged, as described in the Results section. A pellet and supernatant were collected and analysed.

(*a*)

Subfraction	Alkaline phosphatase ...	Specific activity (munits/mg)			Isoenzyme distribution (% within tissue)			Isoenzyme distribution (% within subfraction)	
		Total	Heat-stable	Heat-labile	Total	Heat-stable	Heat-labile	Heat-stable	Heat-labile
Postmicrosomal supernatant		38	38	0.0	46.3	46.3	0.0	100	0
Rough membranes		560	560	0.0	23.7	23.7	0.0	100	0
Smooth membranes		880	880	0.0	29.8	29.8	0.0	100	0
Free polyribosomes		115	84	31	0.2	0.2	100	77	23

(*b*)

Postmicrosomal-supernatant subfraction	Alkaline phosphatase ...	Specific activity (munits/mg)			Isoenzyme distribution (% within postmicrosomal supernatant)			Isoenzyme distribution (% within subfraction)	
		Total	Heat-stable	Heat-labile	Total	Heat-stable	Heat-labile	Heat-stable	Heat-labile
Supernatant		16.8	16.8	0.0	72.1	72.1	0.0	100	0
Pellet		255	255	0.0	27.9	27.9	0.0	100	0

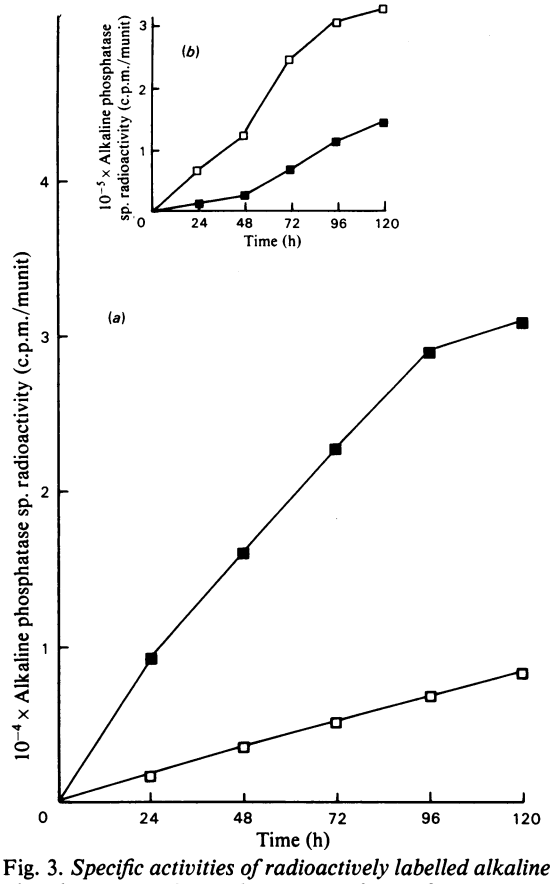

Fig. 3. *Specific activities of radioactively labelled alkaline phosphatase and total protein from first-trimester placenta in organ culture*

Organ culture, alkaline phosphatase assays, protein determinations, SDS/polyacrylamide-slab-gel electrophoresis and radioactivity measurements were performed as described in the Experimental section. (*a*) The specific radioactivities of total alkaline phosphatase (c.p.m. of alkaline phosphatase gel band/munit of enzyme activity of sample applied to gel) in tissue and media were calculated over the time course of organ-culture incubation (120h). Specific radioactivities shown in the media represent additive c.p.m. secreted into the medium up to the given time, divided by additive milliunits of alkaline phosphatase secreted over the same time period. Symbols: □, tissue; ■, medium. (*b*) The specific radioactivities of total protein (c.p.m./mg) in tissue and media were calculated over the 120h organ-culture incubation, with additive values used for media. Symbols: □, tissue; ■, medium.

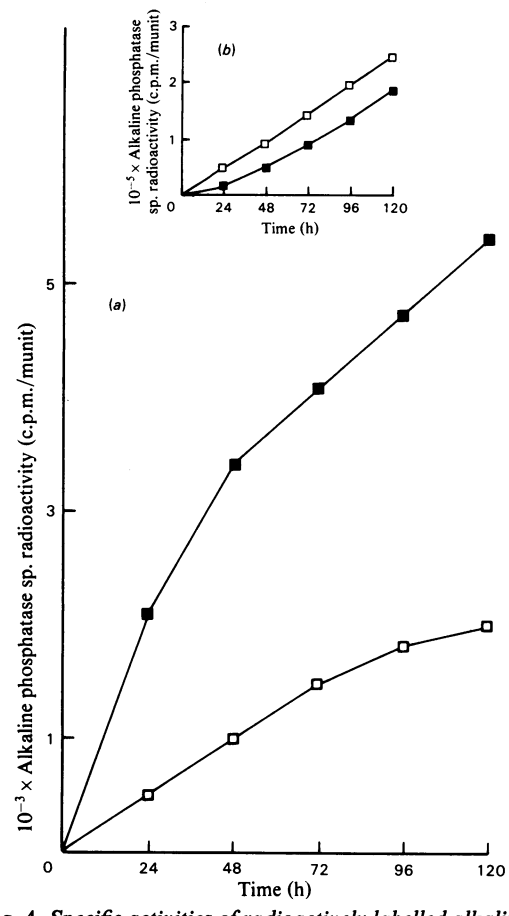

Fig. 4. *Specific activities of radioactively labelled alkaline phosphatase and total protein from term placentas in organ culture*

For experimental details, expression of results, and symbols, see Fig. 3.

Incorporation into total protein for these systems likewise increased with time (see Figs. 3*b* and 4*b*). The first-trimester and term placental tissue thus displayed the same general pattern of synthesis *de novo* and secretion of protein and total alkaline phosphatase with time in organ culture, although the first-trimester tissue was more active than term tissue in this regard.

In first-trimester as in term placenta, the medium showed higher specific radioactivity (c.p.m./munit of enzyme activity) for total alkaline phosphatase than did the tissue (Figs. 3*a* and 4*a*). For total protein, however, specific radioactivities (c.p.m./mg of protein) were far higher in tissue than in medium at corresponding times. Although the corresponding activities of newly synthesized total alkaline phosphatase are lower than those of newly made total protein in these placental systems, specific radioactivities of total alkaline phosphatase cannot be compared with those for total protein, because of the use not only of a different method of radioactive

determination for each, but also of different units for the specific radioactivities of each (see legend to Fig. 3). The experiments in this section were repeated five times with a variation of ± 10%.

Discussion

Placental alkaline phosphatases, members of the group of oncodevelopmental proteins, have been purified and well characterized. In addition, the production of alkaline phosphatases similar to the placental- and liver-type isoenzymes (by enzymic, antigenic and physico-chemical properties) by human carcinoma cell lines in cell culture has been investigated (Hamilton *et al.*, 1979). We have chosen to study the synthesis *in vitro* and secretion of the alkaline phosphatase isoenzymes by normal first-trimester and term placental tissue in organ culture.

Two alkaline phosphatase activities (a heat-stable and a heat-labile activity) were found in both the tissue and incubation media of human first-trimester placenta in our organ-culture system. Two distinct forms of alkaline phosphatase have been separated and characterized in first-trimester placenta (Sakiyama *et al.*, 1979); it seems likely that the two activities evident in our system correspond to the presence of these two alkaline phosphatase isoenzymes. The specific activity of each isoenzyme in the tissue remained constant over the course of incubation (120h), but increased with time in the medium. It appears that a constant tissue pool exists for each alkaline phosphatase (heat-stable and heat-labile) and that synthesis of each isoenzyme in excess of that amount needed to maintain its respective pool is secreted into the medium. Alkaline phosphatase synthesis thus seems to be the driving force for its secretion.

In the tissue the specific activities of the heat-stable and heat-labile alkaline phosphatases were nearly equal, yet the heat-labile isoenzyme was predominant in the medium. If the activity of each alkaline phosphatase isoenzyme directly reflects its absolute amount, then the internal pools of the two isoenzymes are of equal magnitude. Furthermore, the predominance of the heat-labile alkaline phosphatase in the medium throughout incubation suggests its preferential synthesis and secretion by the tissue. However, as the activity of each isoenzyme towards the artificial substrate used in alkaline phosphatase assays (see the Experimental section) is not known, the magnitudes of the internal alkaline phosphatase isoenzyme pools can only be compared on the basis of activity. The possibility of differential proteinase attack on the two different alkaline phosphatases, which would further qualify any proposals about their synthesis and secretion, can probably be discounted, since no proteinase activity could be found on either alkaline phosphatase activity in the tissue or medium of organ culture (see the Results section, 'Synthesis and secretion of alkaline phosphatase by placental tissue in organ culture').

Findings in term-placental organ-culture systems, where only the heat-stable alkaline phosphatase could be conclusively found, supported the model proposed for the alkaline phosphatases in first-trimester systems.

The activity of alkaline phosphatase secreted by placental tissue over the 120h in organ culture far exceeded the constant activity found in that tissue. There was a decline in the secretion rate of each alkaline phosphatase isoenzyme into the media over the time course of organ-culture incubation. The results of control experiments (see the Results section) imply that this decline stems from a decreasing synthetic capacity of the placental tissue rather than from an increasing turnover of alkaline phosphatase in the tissue with time. It is tempting to claim that the placental tissue fatigues (with respect to alkaline phosphatase synthesis) owing to its artificial environment. Yet the decline in rate of synthesis and subsequent secretion by first-trimester tissue is different for each of the alkaline phosphatase isoenzymes; hence the secretion of the two isoenzymes may be regulated by different factors.

The question arises as to whether the virtual disappearance of the heat-labile alkaline phosphatase by the end of gestation reflects direct regulation at the level of gene expression or rather at the level of tissue differentiation (i.e., change in the ratio of syncytial trophoblast to cytotrophoblast; Boime *et al.*, 1978) over the course of gestation.

Despite wide biological variation of the individual placentas utilized in our experiments, there was a high degree of uniformity among different placentas at a given stage of gestation. Hence, although first-trimester placental tissue varied in age from 6 to 12 weeks of gestation, the specific activities of each alkaline phosphatase isoenzyme (range of values $\leqslant 5\%$) and their relative distribution within both tissue and media of organ culture from different placentas were quite uniform. In like manner, there was variation in the duration of gestation for term placentas used in our studies, yet uniformity among these placentas was again observed.

By following the placenta's incorporation of [^{3}H]leucine *in vitro* into its alkaline phosphatases and total protein, their synthesis *de novo* and secretion were confirmed and studied. The specific radioactivities (c.p.m./munit) of alkaline phosphatases in the media were much higher than corresponding values in the tissue throughout organ-culture incubation. If new synthesized alkaline phosphatase first mixed with the pre-existing pool before its secretion, then corresponding specific radioactivities in the media should be less than or

equal to those in the tissue. From this result it can be thus concluded that alkaline phosphatase exists in at least two different metabolic pools within the tissue: one that is directly secreted and one pre-existing pool that is not. Furthermore, under our conditions, mixing between these two pools was not completed within the 120h of organ-culture incubation.

Although the specific radioactivity of total protein increased in both tissue and media of first-trimester systems with time, activities in the tissue far exceeded those in the media. This result is expected when one recalls that protein synthesis *de novo* includes both secretory proteins as well as those destined for structural and/or metabolic roles within the tissue.

Owing to the method of determining radioactive incorporation into alkaline phosphatase in placental systems (see the Experimental section), one cannot exclude the possibility that incorporation into protein of the same apparent molecular weight may interfere with radioactivity measurements on alkaline phosphatase. This difficulty could be avoided by the use of antibodies to precipitate alkaline phosphatase specifically; the radioimmunoassay technique would furthermore allow the separate study of synthesis *de novo* and secretion of each of the alkaline phosphatase isoenzymes.

Notwithstanding the much higher specific activity (munits/mg of total protein) of alkaline phosphatase in term than in first-trimester placental organ-culture systems (about 5-fold), first-trimester placental tissue showed far greater radioactive incorporation (c.p.m./munit) into alkaline phosphatase than did term tissue (about 5-fold) at corresponding times. However, these results can be explained if the heat-stable isoenzyme were much more active towards the substrate used in alkaline phosphatase assays than is the heat-labile isoenzyme. According to this proposal, the lower incorporation in term placenta could reflect the smaller absolute amounts of the heat-stable alkaline phosphatase (and therefore smaller c.p.m. values) needed to cause the activity measured.

We have found alkaline phosphatase to be largely a secretory enzyme in placental tissue; it has also been reported as a plasma-membrane-bound enzyme, which may have a structural function as well as a role in active transport (Fishman & Farran-Furstenthal, 1978). According to current theories on protein synthesis (Redman & Sabatini, 1966), alkaline phosphatases should be synthesized primarily on membrane-bound, as opposed to free, polyribosomes. Subfractionation of human first-trimester placental tissue (postmitochondrial supernatant) yielded a rough-membrane subfraction containing a predominance of the heat-labile alkaline phosphatase activity, similar to that found in the incubation media of first-trimester organ-culture systems; the smooth-membrane subfraction showed nearly the same isoenzyme distribution as the rough membrane. If the rough membrane marks the major intracellular site of alkaline phosphatase synthesis, then the similarity in isoenzyme composition between the rough membrane from fresh tissue and the medium of organ culture strengthens the validity of our system *in vitro* as a first stage in study of synthesis *in vivo* and secretion of placental alkaline phosphatases. Furthermore, the similar distribution of the two alkaline phosphatases in the smooth- and rough-membrane subfractions supports the theory of movement of this secretory protein from its synthesis on the rough membrane, through the smooth membrane, towards eventual secretion from the cell.

The smooth-membrane subfraction prepared in these experiments contained smooth membranes, Golgi elements and plasma membranes. Separation and analysis of the alkaline phosphatases in each of these membranous components is required. Electron-microscopic examination of the rough and smooth membranes revealed a cross-contamination of less than 10%.

Subfractionation of human term placental tissue yielded rough and smooth-membrane subfractions containing only heat-stable alkaline phosphatase. This isoenzyme was likewise the only one confirmed in the tissue and incubation media of term placental organ-culture systems.

After preparation of the rough membrane stripped of its ribosomes and the bound polyribosomes from the rough-membrane subfraction of first-trimester and term placental tissue, the presence of both isoenzymes in each prepared subfraction lent support to the concept of alkaline phosphatase synthesis on the membrane-bound polyribosomes, followed by its vectorial transport into the lumen of the rough endoplasmic reticulum. The finding of alkaline phosphatase on the bound polyribosomes may be at least partly explained by the possible enzymic activity of the alkaline phosphatase monomer which is in the process of being synthesized, or by the formation of an active dimer formed by one complete monomeric peptide and one monomer in the process of chain elongation.

The alkaline phosphatase analyses on the free polyribosomes must be interpreted with caution, for the tiny relative alkaline phosphatase content of this subfraction is near that relative percentage adsorbed to the free polyribosomes in control experiments with ^{32}P-labelled enzyme (see the Results section).

The postmicrosomal-supernatant subfraction contained most of the alkaline phosphatase found in the postmitochondrial supernatant of both first-trimester and term placentas. The relative abundance of alkaline phosphatase in this subfraction can alternatively be viewed as indicative of a metabolic role for the enzyme in regulation of intracellular

metabolism or as an artifact of possible residual membranous components in the supernatant. When subjected to centrifugation, a much higher percentage of the heat-stable activity was found in the pellet obtained from a postmicrosomal supernatant derived from term placental tissue than from that derived from first-trimester tissue.

Alkaline phosphatase activity in the postmicrosomal supernatant may not be simply an artifact, for, despite wide variation in speeds and duration of tissue homogenization before subfractionation, the postmicrosomal-supernatant subfraction showed the same relative amount of alkaline phosphatase in each experiment. In addition, alkaline phosphatase activity in the pellet might be at least partially explained by the high-molecular-weight aggregates of alkaline phosphatase that have been reported in other systems (Sakiyama *et al.*, 1978).

In conclusion, it seems that alkaline phosphatase will be an efficient tool for the study of human gene expression. As with human choriogonadotropin, the synthesis and secretion of which have been investigated in our laboratory (Folman *et al.*, 1980), the production of alkaline phosphatases reflects a change in gene expression during placental development.

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