Protein Phosphorylation in Normal and Neoplastic Development

PHOSPHORYLATION OF PROTEINS ENDOGENOUS TO FOETAL TISSUES AND TUMOURS

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The abilities of proteins endogenous to normal and neoplastic tissues to serve as substrates in a protein-phosphorylation reaction *in vitro* were compared. After the tissue extracts were incubated with [γ - ^{32}P]ATP, the phosphorylated proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the dried gels were subjected to radioautography. Considerable incorporation of ^{32}P into a protein of mol.wt. 135000 was observed with extracts from foetal tissues and tumours, but only minimal incorporation into this protein occurred when extracts from adult tissues were used. The ability of this protein to become phosphorylated *in vitro* may be related to cell proliferation. When ascites cells were incubated with [^{32}P]P_i, one of the major phosphoproteins migrated on sodium dodecyl sulphate/polyacrylamide gels at mol.wt. 135000, suggesting that this protein can be phosphorylated both in intact cells and broken-cell preparations. A protein of mol.wt. 87000 was highly phosphorylatable in extracts from solid tumours, but was not phosphorylated in extracts from ascites tumours, foetal or adult tissues. The phosphorylation pattern of these two proteins can thus distinguish solid neoplasms and normal adult tissues from ascites tumours and from foetal tissues. A protein of mol.wt. 49000, which was the most labelled protein in adult tissues, was also one of the major phosphoproteins in foetal and neoplastic tissues. Numerous mechanisms are postulated to explain how the extent of ^{32}P incorporation into a protein could vary as a function of biological state.

Certain proteins undergo one or more types of 'processing' reactions during their lifetime. Such reactions include limited proteolytic digestion, non-covalent associations with other proteins, vitamins and metals, and covalent modifications such as glycosylation or phosphorylation. All such processing reactions modify the structure of the protein substrates and hence their biological activity. The turnover of phosphate from phosphoserine and phosphothreonine residues is especially interesting because of its transiency, i.e. the net state of phosphorylation of a protein fluctuates in response to environmental changes. Indeed, cyclic AMP mediates the action of several peptide and catecholamine hormones and neurotransmitters by activation of the protein kinase enzymes that catalyse protein-phosphorylation reactions (Kuo & Greengard, 1969). Although cyclic AMP has been the most intensely studied modifier of protein-phosphorylation reactions, other modifier agents have also been described (Kuo & Greengard, 1970). The intracellular agents that regulate most protein-phosphorylation reactions *in vivo* are still unknown.

One of the first studies *in vitro* describing the phosphorylation of proteins endogenous to a tissue was that of Johnson *et al.* (1972). A rat brain synaptosomal extract was incubated with [γ - ^{32}P]ATP, thereby making the extract the source of both the kinase enzymes and the protein substrates, and the resultant phosphorylated proteins were separated on the basis of size by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Subsequent drying of the gel and exposure of X-ray film to these dried gels allowed observation of the phosphorylated proteins as bands on the film. The relative densities and band locations represent a biochemical phenotype that is unique to a given biological preparation (i.e. cell type, subcellular fraction etc.) (Malkinson *et al.*, 1975). We have used this phosphorylation assay *in vitro* to study changes in endogenous protein phosphorylation during cell-cell interactions (Wehner *et al.*, 1977) and to study the effect of a mitogen on lymphocytes (Malkinson *et al.*, 1978). This technique is used in the present paper to show that foetal and adult tissues differ in the phosphorylatability of their endo-

genous proteins, that tumours partially revert to a foetal phenotype of protein phosphorylation and that there is a phosphorylatable protein unique to solid tumour extracts. A preliminary report of some of this work has appeared (Malkinson *et al.*, 1977).

Materials and Methods

Preparation of extracts from tissues and tumours

Neonatal and adult tissues were prepared from A/Umc-strain mice killed by cervical dislocation. Foetal bovine tissues were removed from intact placentae obtained fresh from a local slaughterhouse. Various types of transplantable solid mouse tumours were obtained from Jackson Laboratories (Bar Harbor, MA, U.S.A.). Tumours RB16, MFH and AFH are heavily pigmented, lightly pigmented and unpigmented genetic variants respectively, of B16 melanoma kindly supplied by Dr. J. Sauk, Jr. (University of Minnesota). Three lymphoid ascites tumours (EL-4, P815Y and LSTRA) were kindly supplied by Dr. B. W. Handwerger (University of Minnesota).

Solid tumours were removed after 10–21 days (weight range 300–1000mg) and the capsule and necrotic portions, if present, were discarded before homogenization. Dissected tissues and solid tumours were homogenized in 5 vol. (w/v) of cold unbuffered 0.32M-sucrose by ten strokes with a Teflon pestle in a glass homogenizer. Extracts from ascites-grown tumour cells were prepared as follows. Ascites fluid was removed from mice 5–10 days after intraperitoneal transfer by lavage with 5ml of RPMI 1640 culture medium (Grand Island Biological, Grand Island, NY 14072, U.S.A.). Cells were collected by centrifugation for 3min at 400g, washed three times in 5ml of medium and the final supernatant discarded. To lyse erythrocytes the pellet was resuspended in 2ml of 30mM-Tris/HCl, pH 7.2, containing 0.9% (w/v) NH_4Cl . After 5min at room temperature (22°C), 2ml of RPMI 1640 medium was added and the tumour cells were collected by centrifugation for 3min at 400g. The cell pellet was washed twice in 5ml of unbuffered 0.32M-sucrose and resuspended in sucrose to 5×10^7 cells/ml. Extracts were prepared by sonication with two 10s bursts at a unit setting of 2 of a microtip-equipped Sonifier cell disrupter (Model W185, Heat Systems-Ultrasonics, Plainview, NY 11803, U.S.A.). Initially, protein samples from all extracts were prepared by using the final supernatant (cytosol) from the following consecutive centrifugations: 3000g, 5min; 27000g, 15min; 105000g, 40min. In later experiments the supernatant from a single centrifugation at 40000g for 40min was used and the phosphorylation results were essentially identical.

Assays

The endogenous-protein-phosphorylation assay (Malkinson *et al.*, 1975) contained, in a final volume of 100 μl , 70–140 μg of protein (determined as described by Lowry *et al.*, 1951), 1.0–1.5 μM -[γ - ^{32}P]ATP (sp. radioactivity 5–20 Ci/mol), 10mM- MgCl_2 and 50mM-Mes (4-morpholine-ethanesulphonic acid), pH 6.5. After 3min at 30°C the reaction was terminated by the addition of 50 μl of a sodium dodecyl sulphate/dithiothreitol 'stop' solution (DeLorenzo & Greengard, 1973), and the tubes were boiled for 2min.

To phosphorylate intact ascites cells the following procedure was used. Ascites cells were collected and washed, and the erythrocytes were lysed as described above. The cell pellet was then resuspended to 15×10^6 – 30×10^6 cells/ml of RPMI 1640 medium. Then 50 μCi of carrier-free $\text{H}_3^{32}\text{PO}_4$ (New England Nuclear Corp., Boston, MA, U.S.A.) was added to the cells and the suspension incubated at 37°C for 2h. After incubation the cells were pelleted by centrifugation at 400g for 20min, resuspended in 0.25M-sucrose containing 1% sodium dodecyl sulphate and boiled for 3min. The resulting cloudy suspension was cleared by homogenization with six strokes of a Teflon/glass homogenizer. This solution was diluted to 1mg of protein/ml with the 'stop' solution.

Fractionation of phosphorylated substrates from the phosphorylation reactions *in vitro* and with intact cells by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis, subsequent radioautography and measurement of [^{32}P]P_i incorporation by microdensitometry were carried out as described previously (Ueda *et al.*, 1973).

Results

Characteristics of endogenous protein phosphorylation in adult tissues

The phosphorylation pattern of eight different tissues from adult mice (Fig. 1) is consistent with the fact that some proteins are common to all tissues, whereas other proteins are tissue-specific. The pattern of proteins stained with Coomassie Blue bears no relationship to the phosphoprotein profile, suggesting that many of the endogenous proteins that can be phosphorylated under these assay conditions are present in tissues in relatively small amounts. The ability of proteins to serve as phosphorylatable substrates depends in part on the bivalent cations included in the assay, as noted previously (Malkinson *et al.*, 1975). The mechanism by which cations modify the substrate specificity of protein kinases is unknown. The only protein that was phosphorylated in the presence of each cation in all tissues migrated at the 49000-mol.wt. region of the gel (protein 49). For most

Fig. 1. *Phosphorylation of proteins endogenous to adult mouse tissues*

Cytosols from eight tissues (Li, liver; Lu, lung; K, kidney; T, testis; S, spleen; H, heart; B, brain; F, epididymal fat-pad) were prepared as described in the Materials and Methods section. Each sample was phosphorylated in the presence of (a) 10 mM-MgCl₂, (b) 5 mM-ZnCl₂ or (c) 5 mM-CaCl₂. The phosphorylated samples were then applied to sodium dodecyl sulphate/polyacrylamide-slab gels and subjected to electrophoresis. The direction of electrophoresis in this and all subsequent radioautograms was downward towards the anode. The gels were stained, dried and subjected to radioautography as described in the Materials and Methods section. The protein-staining pattern of the gel is shown in (d).

tissues this protein is the major phosphoprotein observed on the gel. We have suggested previously that a protein of mol.wt. 49000 from rat and bovine tissues may be the regulatory subunit of a cyclic AMP-dependent protein kinase enzyme (Malkinson *et al.*, 1975).

Developmental changes in protein phosphorylation

When the phosphorylation of proteins endogenous to mouse neonatal and adult tissues was compared (Fig. 2) it was observed that the extent of phosphorylation changed markedly with age. The phosphorylatability of brain, spleen and lung proteins increased with age, whereas that of liver and heart proteins decreased. The extent of kidney

protein phosphorylation varied somewhat from experiment to experiment.

In only one case did a protein undergo an age-dependent change in the extent of phosphorylatability that differed from that of the other proteins of that tissue. The phosphorylatability of a lung protein with mol.wt. 135000, when assayed in the presence of Mg²⁺, decreased with age, whereas the phosphorylatability of the other lung proteins increased. When the amount of ³²P incorporated into this protein was measured by using lung extracts from mice of different ages (Fig. 3), protein phosphorylation was greatest in foetal mice. To determine whether this phosphoprotein was tissue-specific to lung and species-specific to mice, the

Fig. 2. Phosphorylation of proteins endogenous to neonatal and adult mouse tissues

Tissues were dissected from neonatal (N) mice of 1–4 days of age or from adults (A) that were 6–8 weeks old. Soluble fractions were prepared and subjected to phosphorylation in the presence of (a) 10mM-MgCl₂, (b) 5mM-ZnCl₂ or (c) 5mM-CaCl₂. Electrophoretic separation of the resultant phosphorylated proteins and radioautography of the dried gels were carried out as described in the Materials and Methods section. The tissues used were: B, brain; Li, liver; Lu, lung; S, spleen; K, kidney; H, heart. The arrow indicates the position of protein 49 in each gel.

Fig. 3. Amount of phosphate incorporated into a mouse lung protein of mol.wt. 135000 as a function of age

Lung cytosol was prepared from mice of different ages as described in the Materials and Methods section. Equal amounts of protein from each sample were then phosphorylated in the presence of 10mM-MgCl₂ and subjected to electrophoresis and subsequent radioautography. The amount of ³²P incorporated into the 135000-mol.wt. region of the gel was determined by microdensitometric tracing of the radioautogram. The data are expressed as the relative percentage of the band density at this molecular-weight position from lung samples taken from mice at each age compared with the 135000-mol.wt. band density of lung cytosol from 0–1-day-old mice.

phosphorylation of various foetal bovine tissues was examined (Fig. 4): in all of these, a protein of mol.wt. 135000 was the major phosphoprotein. This is in distinct contrast with adult tissues, where most ³²P is incorporated into protein 49 under these assay conditions.

Protein phosphorylation in solid tumours

Since a highly phosphorylatable protein of mol.wt. 135000 is a foetal characteristic, the phosphorylation of extracts from solid tumours was examined for the presence of this foetal phenotype. A typical radioautogram is shown in Fig. 5 and the data are summarized in Table 1, indicating results from 27 different transplantable tumours, including five sarcomas, four leukaemias and six carcinomas of different origins. The tumours ranged from well-differentiated (rhabdomyosarcoma) to relatively undifferentiated (anaplastic carcinoma) and also had a range of growth rates. There were typically three major phosphorylated proteins in these tumours: protein 49, and bands which migrated at mol.wts. 135000 and 87000. Only four of the 27 solid tumours tested lacked a heavily phosphorylatable protein

Fig 4. *Phosphorylation of proteins endogenous to foetal bovine tissues*

The following tissues were moved from a bovine foetus (40 cm long, 5.4 kg in weight): lung (Lu), liver (Li), spleen (S), thyroid (Tr) and thymus (Tm). Cytosol fractions were prepared, phosphorylated in the presence of 10 mM-MgCl₂ and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The gels were then stained, destained, dried and exposed to X-ray film. These procedures are described in the Materials and Methods section. The arrows indicate the positions of 135 000- and 49 000-mol.wt. proteins on the gels.

of mol.wt. 135 000. A highly phosphorylated protein of mol.wt. 135 000 has also been observed by us in various transformed cultured cell lines, including C6 glioma, T3 schwannoma, and SV40-transformed 3T3 cells (Wehner *et al.*, 1977). Actively growing normal (untransformed) 3T3 cells and human diploid fibroblasts also contained a highly phosphorylatable protein of mol.wt. 135 000 (Wehner *et al.*, 1977). Highly phosphorylated protein 49 has been observed in every foetal, adult, tumour and cultured cell line examined in this laboratory (Malkinson *et al.*,

1975; Wehner *et al.*, 1977), as well as by other workers (Rubin & Rosen, 1973; de Jonge, 1976). The protein of mol.wt. 87 000 has not been previously observed in foetal or adult tissues or in transformed cell lines.

Ascites-cell protein phosphorylation

Since biochemical phenotype sometimes depends on the physical state of the tumours (Kozak & Murphy, 1976), we compared the phosphorylation of ascites and solid tumours developed from the

Fig. 5. *Phosphorylation of soluble protein extracts from solid mouse tumours*

Extracts were prepared from various solid tumours as described in the Materials and Methods section. The phosphorylation reaction was performed in the presence of 10 mM-MgCl₂ and the samples subjected to electrophoresis and subsequent radioautography. Abbreviations: AC, anaplastic carcinoma 15091 AK; SS, spindle-cell sarcoma SaI; LS, lymphosarcoma 6C3HED; RS, rhabdomyosarcoma BW10139; A and N refer to phosphorylated lung cytosol markers taken from adult and neonatal mice respectively; NB, neuroblastoma C1300; PS, pleiomorphic sarcoma S180. The arrow indicates the position of the 135000-mol.wt. proteins on the gels.

same tumour-cell types (Fig. 6, Table 1). Both protein 49 and the protein of mol.wt. 135000 were phosphorylated in ascites and solid tumours. No phosphorylation of the protein of mol.wt. 87000, however, was apparent when extracts from ascites cells were used.

To determine whether any of the proteins phosphorylated in broken-cell preparations were also phosphorylated in intact cells, the following experiment was performed. Intact ascites cells were incubated with [³²P]P_i and the pattern of proteins

labelled with phosphate was compared with that obtained by the reaction *in vitro* (Fig. 6). The two most heavily phosphorylated proteins observed with the intact EL-4 lymphoma shown in Fig. 6 migrated at *R_F* values corresponding to mol.wts. 180000 and 135000. This was also true for the other intact ascites tumours listed in Table 1 as well as for intact normal mouse spleen lymphocytes (results not shown).

A minor phosphorylated band that co-migrated with protein 49 was also observed with all intact-cell

Table 1. *Protein-phosphorylation patterns in tumours*

Extracts were prepared from transplantable tumours and subjected to the protein-phosphorylation assay in the presence of 10mM-MgCl₂ as described in the Materials and Methods section. The plus signs refer to degrees of phosphorylation (+++, heavily phosphorylated; +, slightly phosphorylated) estimated by visual observation. A minus sign indicates the lack of any phosphorylation. At least two separate experiments with tumours freshly transplanted in this laboratory at different times were carried out with each tumour type. Results were generally quite consistent. In those cases where replicate experiments differed by more than one plus sign, at least four experiments were performed and the average is shown. In no instance was a protein heavily phosphorylated in one experiment and then not at all phosphorylated in another.

Code	Tumour type	Relative amount of phosphorylation			
		Mol. wt. ...	135000	87000	49000
Solid tumours					
SaD2	Fibrosarcoma		++	+	++
S180	Pleiomorphic sarcoma		++	+++	+
6C3HED	Lymphosarcoma		+++	+++	+
SaI	Spindle-cell sarcoma		+++	+++	++
BW10139	Rhabdomyosarcoma		-	+++	+++
BW5147	Lymphatic leukaemia		+++	+++	++
P1534	Lymphatic leukaemia		-	++	+
EL4	Lymphoma		+++	++	+++
LSTRA	Lymphoma		++	+++	+
P815Y	Mastocytoma		-	+++	+
C1300	Neuroblastoma		+++	+++	+++
15091AK	Anaplastic carcinoma		+	+++	+++
RB16	Melanotic melanoma		+	+	++
AFH	Amelanotic melanoma		++	+	++
MFH	Melanotic melanoma		+	+	+
BW6437	Haemangioendothelioma		+	+++	+++
CaD2	Mammary adenocarcinoma		+++	++	++
CaD1	Mammary adenocarcinoma		++	+	+
T1703	Mammary adenocarcinoma		+	+	+
T1699	Mammary adenocarcinoma		++	++	+
H2712	Mammary adenocarcinoma		++	++	+
C3HBA	Mammary adenocarcinoma		++	+	+++
LL1	Lewis lung carcinoma		++	+++	+
LL2	Lewis lung carcinoma		+	++	+
LL3	Lewis lung carcinoma		++	+++	++
LL4	Lewis lung carcinoma		+	+++	+
LL5	Lewis lung carcinoma		-	+++	++
Ascites tumours					
EL4	Lymphoma		++	-	+
LSTRA	Lymphoma		++	-	++
P815Y	Mastocytoma		+	-	+
6C3HED	Lymphosarcoma		+	-	+
SaI	Splindle-cell sarcoma		+++	-	++
BW5147	Lymphatic leukaemia		+++	-	++

preparations used. A minor phosphorylated band at mol.wt. 87000 was sometimes observed with intact ascites-tumour lines.

Discussion

To interpret these results best it is necessary to examine the factors that determine the extent of ³²P incorporation into a protein, and hence the possible mechanisms by which variations in the degree of phosphate incorporation into a single

protein arise. It should first be stated that sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins is not complete, i.e. several proteins can have the same molecular weight and thus appear in the same band on the gel. The 135000 mol.wt. region of the gel is probably heterogeneous and it is possible that the phosphorylated proteins with this *R_f* value found in neuroblastoma and foetal bovine thymus extracts are not identical. Application of two-dimensional electrophoretic techniques should resolve this. However, the association

Fig. 6. Protein phosphorylation in intact EL-4 ascites cells and in extracts of ascites and solid tumours derived from EL-4 ascites cells

Ascites tumours were prepared by intraperitoneal transfer of EL-4 ascites cells, and solid tumours propagated by subcutaneous injection of EL-4 ascites cells. Extracts from the solid (a) and ascites (b) tumours were prepared, phosphorylated in the presence of 10 mM-MgCl₂ and subjected to electrophoresis and radioautography, as described in the Materials and Methods section. Intact ascites cells were collected, incubated with ³²P and then treated as described in the Materials and Methods section to give rise to the radioautogram shown in (c). The arrows indicate the positions on the gel corresponding to mol.wts. 135 000, 87 000 and 49 000.

of the degree of phosphorylation of the protein of mol.wt. 135 000 with cell proliferation, the appearance of a highly phosphorylated protein of mol.wt. 87 000 solely in solid tumour extracts and the universality of protein 49 suggests a widespread distribution of these same proteins among different sources.

The phosphorylatability of proteins in tissue extracts can change because of variations in the concentration of sites available for phosphorylation and because of variations in the activity of the

enzymes (protein kinases and phosphoprotein phosphatases) that catalyse phosphorylation/dephosphorylation reactions. The concentration of phosphorylatable sites depends on the concentration of the apoprotein substrate, the steric accessibility of phosphorylatable sites and whether these sites are already occupied by phosphate groups at the time of assay. For example, there may be a greater concentration of the highly phosphorylatable protein of mol.wt. 135 000 in foetal tissues than in adult tissues, since substantial alterations in the concentration of various proteins occur during maturation (Greengard, 1971), or this polypeptide may be folded in different ways in foetal and adult tissues such that phosphorylatable sites are accessible to foetal protein kinase enzymes, but are 'buried' in the adult extract. Lysozyme in its native configuration cannot be phosphorylated, but after denaturation a phosphorylatable site was exposed (Bylund & Krebs, 1975). A certain amino acid sequence has been found repeatedly at the phosphorylated site of several diverse protein substrates, which consists of an arginine residue located 2-4 amino acid residues towards the N-terminal end of the reactive serine or threonine sites (Kemp *et al.*, 1975). Foetal and adult isoenzymes could exist that differ in the number of these reactive sites. Finally, if the site(s) has already been phosphorylated *in vivo* and this phosphate is not removed during sample preparation, the occupied site will be unavailable for reaction with [γ -³²P]ATP. It has been proposed, for example, that bovine heart cyclic AMP-dependent protein kinase enzymes, which can undergo an autophosphorylation reaction wherein these enzymes phosphorylate themselves, retain their phosphate groups in crude extracts, but gradually lose this phosphate, owing to the action of phosphatase enzymes, during purification (Erllichman *et al.*, 1974).

There is a great heterogeneity of kinase (Corbin *et al.*, 1975) and phosphatase enzymes (Khandelwal *et al.*, 1976). The amount and type of isoenzyme can vary with age (Lee *et al.*, 1976), state of malignancy (Criss & Morris, 1973), stage of the cell cycle (Costa *et al.*, 1976), etc. The state of activation or inactivation of a given enzyme can vary with the physiological status (e.g. the presence or absence of certain hormones) of the animal, and the enzyme can remain in this state during the preparation of crude extracts (Soderling *et al.*, 1973). Various compounds can stimulate or inhibit these enzymes, among them cyclic nucleotides (Kuo & Greengard, 1969), certain heat-stable protein modulators (Ashby & Walsh, 1972) and MgATP²⁻ (Haddox *et al.*, 1972). The concentration of some of these factors has been shown to vary with age (Weiss & Strada, 1973) and with malignant state (Ryan & Heidrick, 1974). As shown in Fig. 1, bivalent cations can alter the substrate specificity

Table 2. Factors governing the phosphorylatability of proteins in endogenous phosphorylation reactions in vitro

- Number of sites
- Concentration of substrate proteins
- Steric accessibility of phosphorylatable site(s) to kinase enzymes
- Prior phosphorylation and dephosphorylation in vivo
- Activity of kinase/phosphatase enzymes
 - Concentration of the kinase and phosphatase enzymes that can use this protein as a substrate
 - State of activation or inactivation of these enzymes at the time of assay
 - Presence during assay of compounds that can modify activities of these enzymes (e.g. cyclic AMP)

Table 3. Characteristic major phosphoproteins in foetal and adult normal tissues and in solid and ascites tumours

The Table represents a summary of the experiments performed, illustrating the trends of different types of tissue samples with regard to the phosphorylatability of two proteins. A given sample may show minor deviations from these trends (see Table 1).

Mol.wt.	...	Relative amount of phosphorylation	
		135000	87000
Foetal tissue		+++	-
Adult tissue		+	-
Solid tumour		++	+++
Ascites tumour		++	-

of these enzymes. Any one or more of these factors (summarized in Table 2) could be responsible for the differences in phosphorylatability of the three proteins discussed here.

Several laboratories have reported differences in the extent of phosphorylation of chromatin proteins between normal and neoplastic tissues. In virus-transformed fibroblasts there is a much greater degree of phosphorylation of several chromosomal proteins than in normal cells (Pumo *et al.*, 1976). Several non-histone proteins were phosphorylated in rat liver, but not in Novikoff hepatoma, and vice versa (Letnansky & Wenzel, 1977). The degree of phosphorylation of different sites on a single type of histone differed between normal and malignant liver (Ganpath *et al.*, 1977). The extent of phosphorylation of this histone type has been positively correlated with tumour growth rate (Balhorn *et al.*, 1972).

Three soluble phosphoproteins have been described in the present paper. Evidence has been presented (Malkinson *et al.*, 1975) that suggests that protein 49 is a protein kinase regulatory subunit. Both its phosphorylation and dephosphorylation are controlled by cyclic nucleotide-bivalent cation interactions (Malkinson *et al.*, 1975; Malkinson, 1975). The extent to which cyclic AMP affects the

phosphorylation and dephosphorylation of protein 49 changes with age (Malkinson, 1977).

A highly phosphorylatable protein of mol.wt. 135000 is found in extracts from foetal tissues, tumours and dividing lymphocytes. Only slight ³²P incorporation into this protein is observed with extracts from adult tissues or resting lymphocytes. The phosphorylatability of this protein is apparently related to proliferation. In mitogen-stimulated lymphocytes the phosphorylation of this protein of mol.wt. 135000 was maximal at the time of maximum DNA synthesis (Malkinson *et al.*, 1978). It would be interesting to understand the relationship between the phosphorylation state of this protein and the regulation of cell division. It would also be interesting to examine this phosphorylation during tumour regression.

A protein of mol.wt. 87000 is the major site of ³²P incorporation in extracts from solid tumours. Its unique distribution, found by assays *in vitro*, suggests that it may be a protein found only in tumour stroma. The degree of phosphorylatability of this protein did not vary with tumour size over a 3-4-fold range of lymphoma sizes. It is not known whether any difference exists between the phosphorylation characteristics of this protein in extracts from benign or malignant tumours.

Another major phosphoprotein that we observed in some experiments was of mol.wt. 110000. It was highly phosphorylated in foetal rat tissues (results not shown), but was absent from foetal bovine tissues. It was consistently more heavily phosphorylated in ascites tumours than in solid tumours (see Fig. 6). However, the degree of its phosphorylation in ascites tumours varied considerably from experiment to experiment. This variation could be explained by our previous report (Wehner *et al.*, 1977) that the phosphorylatability of a protein of mol.wt. 110000 in cultured mammalian cell lines varied with cell density. Tumour cells growing in the peritoneal ascites fluid tend to clump and form aggregates of widely varying size, and this variation in cell-cell contacts may account for the variation in the phosphorylatability of this protein.

Whatever the mechanisms that govern the phosphorylatability of the proteins of mol.wts. 135000 and 87000, the degree of their phosphorylation is characteristic of certain biological states (Table 3). The protein-phosphorylation profiles *in vitro* of solid neoplasms and normal adult tissues distinguish them from ascites cells and foetal tissues. This finding may prove of value in future ontogenetic and oncogenetic studies.

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