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Changes in Microtubule Phosphorylation during Cell Cycle of HeLa Cells

(mitosis/phosphotubulin/protein phosphokinase/tubulin/vinblastine)

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ABSTRACT **The phosphorylation *in vitro* and *in vivo* of tubulin isolated from HeLa cells has been examined during the cell cycle. The results obtained indicate that: (*a*) the protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) activity present in the microtubules, and measured *in vitro* with exogenous casein as substrate, is maximal in M cells, whereas that present in the cytosol is nearly constant during the S, G_2, and M stages, and decreases during G_1; (*b*) the patterns through the cell cycle of the maximal number of tubulin sites phosphorylated *in vitro* and of the microtubular protein kinase activity are similar; (*c*) the degree of tubulin phosphorylation *in vivo* is 2- to 3-fold higher in the microtubules isolated from the S and M stages of the cell cycle, than those from G_1 and G_2. This variable phosphate content of tubulin through the cell cycle suggests that such covalent modification might be important to enable tubulin to carry over some of its functions during the cell cycle.**

It has been recently established that tubulin from different mammalian tissues contains covalently bound phosphate (1–5). It is presently unknown, however, whether the phosphorylated state of tubulin is the only one normally found in this protein, or whether a phosphorylation–dephosphorylation process might play a role in the regulation of the assembly and/or function of the microtubular structure.

One of the most relevant situations in which microtubule assembly–disassembly occurs is during the cell cycle, since tubulin is the fundamental component of the mitotic spindle (6, 7). It is already known that tubulin from HeLa cells can be phosphorylated *in vitro* as well as *in vivo* (5), and we, therefore, investigated the degree of this phosphorylation during the cell cycle of synchronized HeLa cells. The results obtained indicate that tubulin isolated from cells in the S and M stages of the cell cycle contains 2- to 3-fold more covalently bound phosphate than that isolated from the G_1 or G_2 stages.

MATERIALS AND METHODS

Cell Cultures. HeLa cells were seeded at 10^7 cells per Roux bottle (150 cm^2) and cultured in Hanks-lactoalbumin hydrolyzate, supplemented with 5% calf serum, 2.5 units/ml of penicillin G, 2.5 μg/ml of streptomycin, and 20 units/ml of mycostatin ("complete medium"). After 18 hr of exponential growth two different methods of synchronization were used.

Synchronization Techniques. HeLa cells were synchronized either by a double thymidine block (8) (Method I) or by a single thymidine block followed by mitotic accumulation in the presence of colchicine (Method II). When Method I was used, cells were exposed to complete medium containing 2 mM thymidine for 18 hr, to complete medium alone for the following 9 hr, and again to 2 mM thymidine for an additional 18 hr. At the end of this second block, the cells were rinsed and cultured in complete medium until trypsinized at the indicated times.

Defined cell populations were obtained by Method II which consisted in a thymidine block for 24 hr followed by a 6 hr release in complete medium. Colchicine (0.23 μg/ml) was then added, and 6–8 hr afterwards the mitotic (M) cells were detached by gentle rocking with culture medium. The remaining attached cells (G_2) were then trypsinized as usual. Trypsinized cells were washed by resuspension and centrifugation in phosphate-buffered saline (PBS) and stored frozen at −25° until sonicated.

Synchronization was assessed by pulses of [^{3}H]thymidine and scoring of mitotic figures. For this purpose, cells were incubated for 15 min with 1 μCi/ml of [^{3}H]thymidine (40 Ci/mmol, Produits Radioactifs). The incubation was stopped by rinsing twice with cold PBS followed by rapid trypsinization. The detached cells were immediately precipitated with 10% trichloroacetic acid and dissolved in 0.2 N KOH. This operation was repeated twice, the final pellet dissolved in 0.1 ml of concentrated formic acid, and counted in a scintillation counter. Mitotic cells were determined from trypsinized cells which had been fixed in methanol–acetic acid (3:1) and stained with 1% aceto-orcein solution (9). The number of mitotic figures was scored in at least 500 cells at each time point.

Microtubular Fraction Preparation. The frozen HeLa cells were thawed, resuspended (10^8 cells per ml) in 0.01 M phosphate buffer (pH 6.5) containing 0.01 M $MgCl_2$ and 0.24 M sucrose, and sonicated three times at 0–5° for 15 sec each. The suspension was centrifuged at 80,000 × *g* for 2 hr, and the microtubular protein isolated from this cytosol by the vinblastine procedure, as already described (5). Recoveries, as judged by colchicine binding, were 80–90% throughout the cell cycle. Polyacrylamide gel electrophoreses, in the presence of sodium dodecyl sulfate (5), of the microtubular fractions isolated from the HeLa cells synchronized either by Method I or II reveal that, in all instances, more than 90% of the protein migrates as a band of molecular weight of 55,000.

Protein Kinase (ATP:Protein Phosphotransferase, EC 2.7.1.37) Assay. Cytosol (40–60 μg) or microtubular fraction (5–10 μg) was incubated for 30 min at 30° in the presence of

Abbreviations: PBS, phosphate-buffered saline; M, mitotic cells; G_1, cells from the pre-DNA synthetic period; G_2, cells from the post-DNA synthetic period; S, cells from the period of DNA synthesis; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

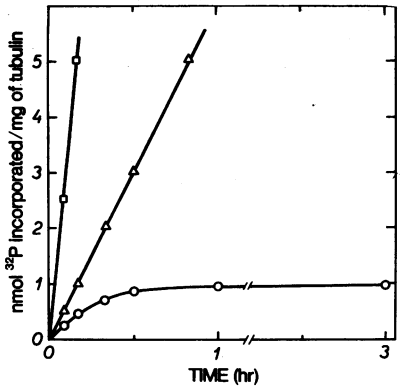

FIG. 1. Protein kinase activity and endogenous phosphorylation of the microtubular fraction of HeLa cells. Exponentially growing HeLa cells were trypsinized, sonicated, and the microtubular fraction isolated by vinblastine precipitation. Aliquots were assayed for protein phosphokinase activity in the presence of casein (□), or histone (△), and for endogenous phosphorylation (○). Other details are indicated under *Materials and Methods*.

casein (11 mg/ml) and 7×10^{-6} M 3′:5′-cyclic AMP, under the conditions described by Piras *et al.* (10). The activity obtained for both fractions was similar regardless of whether the cells (unsynchronized) had been frozen or not.

Tubulin Phosphorylation In Vitro. The standard reaction mixture for tubulin phosphorylation *in vitro* has the following composition: 10–20 μg of microtubular fraction, 30 mM sodium glycerophosphate (pH 6.5), 1 mM NaF, 0.35 mM EGTA, 10 mM magnesium acetate, 0.25 mM [γ-^{32}P]ATP (10^9 cpm/μmol) in a final volume of 0.1 ml. Incubations were carried out for 2 hr at 30° and stopped by the addition of 25 μl of 0.1 M EDTA containing 50 mg/ml of albumin, followed by 2 ml of 5% trichloroacetic acid. The radioactive protein was then processed as described by Piras *et al.* (10) and extracted with butanol saturated with water (5).

^{32}P-Incorporation in Intact Cells. HeLa cells were cultured in complete medium for 18 hr after seeding, carefully rinsed three times with "phosphate-deficient medium" (a "complete medium" from which the phosphate salts of the Hanks' solution were omitted), and exposed to a phosphate-deficient medium containing carrier free $^{32}P_i$ (1 μCi/ml). Cells were

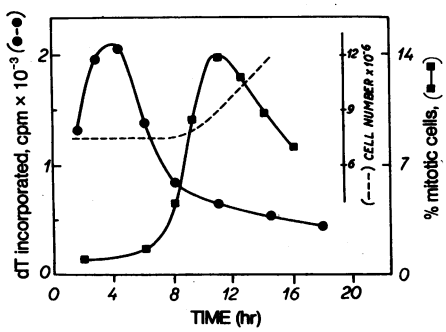

FIG. 2. Changes in [^{3}H]thymidine incorporation into DNA, mitotic index, and cell number at different stages in the cell cycle of HeLa cells. The cells were synchronized by a double thymidine block (Method I), trypsinized at the times indicated on the abscissa (the release from the second thymidine block is the zero time), and aliquots analyzed for the parameter shown, as described under *Materials and Methods*.

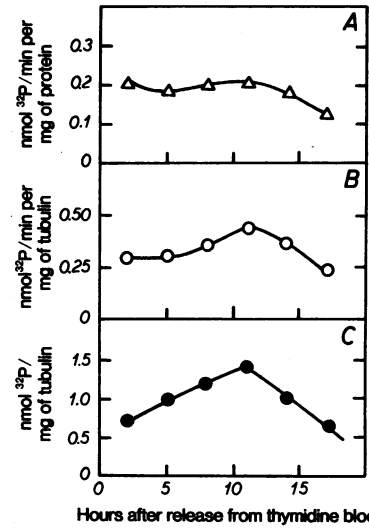

FIG. 3. Protein kinase activity and endogenous phosphorylation at different stages in the cell cycle of HeLa cells. Cells were obtained after release from a double thymidine block (Method I) at the times indicated in the abscissa, and the cytosol and microtubular fraction prepared as described under *Materials and Methods*. Protein kinase activity was measured under the standard conditions in the cytosol (A) and in the microtubular fraction (B), and the endogenous phosphorylation was measured in the microtubular fraction (C).

cultured in this radioactive medium for another 35–40 hr as well as during the following period of synchronization (Method II) and until they were trypsinized at the different stages of the cell cycle. The collected cells were rinsed twice with cold PBS, and the microtubular fraction isolated. Its radioactivity was determined after repeated precipitation with trichloroacetic acid in the presence of carrier albumin, followed by three extractions of the pellet with *n*-butanol saturated with water, in order to remove bound phospholipids. The radioactivity associated with the tubulin peaks on gel electrophoreses after *in vitro* or *in vivo* labeling was approximately 70% of that determined directly after the butanol extraction, and 80–85% of that present in all of the gel. These figures were independent of the stage of the cell cycle from which the material was derived.

Protein Determination. Protein was measured according to Lowry *et al.* (11) after trichloroacetic acid precipitation of the samples and methanol extraction of the pellet.

Materials. Casein (Fisher Scientific Co.) was treated according to Reimann *et al.* (12) before use. Colchicine and histone (type II-A) were purchased from Sigma Chemical Co. (St. Louis). [γ-^{32}P]ATP was prepared enzymatically (13, 14). Vinblastine-sulfate was a generous gift from Eli Lilly Co. and griseofulvin was kindly supplied by Laboratorios Glaxo (Argentina) S.A.C. e I.

RESULTS

The characteristics of the phosphorylating activity present in the vinblastine-precipitate obtained from the soluble fraction of exponentially growing HeLa cells was investigated. As has been demonstrated for other tissues (1–5, 15), the microtubular fraction from HeLa cells has a protein phosphokinaes activity which is capable of phosphorylating exogenous substrates (casein, histone, phosphovitin, etc.) as well as tubulin.

TABLE 1. *Protein kinase activity and endogenous phosphorylation of the microtubular and cytoplasmic fractions of HeLa cells at different stages of the cell cycle*

Cell stage	Cytosol	Microtubular fraction			
	Protein kinase activity (casein) (pmol/min per mg)	Protein content (μg/10^6 cells)	Protein kinase activity (casein) (pmol/min per mg)	Endogenous phosphorylation (pmol/mg)	
				(tubulin)	(diglyceride)
S	192	2.9	343	720	700
G_2	180	3.7	419	1470	859
M	169	2.6	1009	5336	996
G_1	81	3.0	305	499	944

HeLa cells were synchronized and obtained by Method II. The M cells were 95% pure and the G_2 were cross-contaminated only to the extent of 3%. S and G_1 cells were obtained by trypsinization of cultures at 2 and 17 hr, respectively, after release from a single thymidine block. In order to equalize the conditions as much as possible to those used for obtaining M and G_2 cells, we added colchicine 15 min prior to trypsinization also to the S and G_1 cells. The purity of these cells was estimated to be 93% and 80–85%, respectively, and was determined by the method of Puck (8) for scoring thymidine-labeled and mitotic cells. The protein content indicated in the table was obtained by direct measurements (11), but it is thought to represent the actual tubulin level because of the high recovery of colchicine binding upon vinblastine precipitation and the purity of the fractions determined by gel electrophoreses (see *Materials and Methods*). Other experimental details are indicated under *Materials and Methods*.

Fig. 1 indicates that a substantial endogenous phosphorylation takes place in the presence of [γ-^{32}P]ATP; the reaction rate is nearly linear for 15 min and reaches a plateau between 0.5 and 1 hr. Hence, the maximal level of tubulin phosphorylation *in vitro* was determined in all subsequent experiments after 2 hr incubation. On the other hand, the tubulin concentration present in the assays is probably not saturating, and therefore, the determination of the actual protein kinase activity present in the microtubular fraction is better achieved by the use of an excess of an exogenous substrate. The results obtained indicate that, in fact, the reaction rates with exogenous substrates are higher and linear for at least 1 hr. Casein is the best of the three substrates tested; phosphovitin is only one-half as efficient and histone one-fourth as efficient. Protein kinase activity was, therefore, measured in all subsequent experiments as the initial rate of casein phosphorylation. Adenosine 3′:5′-cyclic monophosphate increases the phosphorylation of either tubulin or the exogenous substrates by only 10–20%. It is important to note that this microtubular protein kinase differs from the activity of the corresponding cytoplasmatic fraction both in substrate specificity and in the activation by cyclic-AMP.

Protein kinase activity and endogenous phosphorylation of tubulin were examined in synchronously growing HeLa cells at different times after release from a double thymidine block. The degree of synchrony obtained by this procedure is illustrated in Fig. 2. The S phase as measured by the rate of DNA labeling with [^{3}H]thymidine, begins immediately after the release from the thymidine block, and lasts for 6–8 hr with a maximum at 4 hr. The highest percentage of mitotic cells (14%) occurs 12 hr after the release from the thymidine block, but the peak is relatively broad (6–8 hr). An increase in cell number is first evident at about 9 hr, and by 14–16 hr the population has almost doubled. These data indicate that the parameters of the cell cycle of our cultures are similar to those reported for HeLa cells by other authors (9, 16).

The protein phosphokinase activity, measured as the rate of casein phosphorylation (Fig. 3B), and the maximal tubulin phosphorylation (Fig. 3C) of the microtubular fraction of cells harvested at different times after the release from the thymidine block, have been compared with the cytoplasmic protein phosphokinase activity (Fig. 3A). The results obtained indicate that (*a*) the increase of protein phosphokinase activity of the microtubular fraction during late G_2 and M (8–14 hr) does not parallel any detectable change in the cytoplasmic fraction, and (*b*) that an increased maximal phosphorylation of tubulin is also observed in late G_2 and M. This coincidence, however, is not necessarily due to the microtubular protein phosphokinase activity pattern, since the endogenous phosphorylation assays were carried out until the plateau of phosphorylation was reached.

Although the changes observed in protein phosphokinase activity and endogenous phosphorylation were distinct and reproducible, it was considered that the apparently small differences observed were probably due to the mixed population of G_2 and M cells which coexist between 8 and 12 hr after the release from the thymidine block. In fact, no more than 15–20% of mitotic cells were present at any time. Therefore, another method of obtaining better defined cell populations was used. It consisted of a combination of a single thymidine block followed by mitotic selection in the presence of colchicine (Method II). The results obtained in a typical experiment with this procedure are indicated in Table 1. It can be observed that the protein phosphokinase of the cytoplasmic fraction remains nearly constant during S, G_2, and M, but drops significantly during G_1. This behavior is consistent with the pattern observed in Fig. 3A and with the data reported for HeLa cells by Karn *et al.* (16) who used histone as substrate. The specific activity of the protein phosphokinase of the microtubular fraction shows a 3-fold increase in the mitotic cells and a minor but consistent increase also in the G_2 cells. It should be noted that this specific activity is expressed in terms of tubulin. Therefore, the observed variations through the cell cycle indicate that the phosphokinase associates with changing stoichiometry to the microtubules, and that it is a different entity than tubulin. The protein kinase activity present in microtubular preparations and the colchicine binding activity (tubulin dimer) have been separated in other systems (4, 5).

Since we have recently observed that two components were involved in the endogenous phosphorylation *in vitro* of the microtubular fraction, the ^{32}P-incorporation was studied not

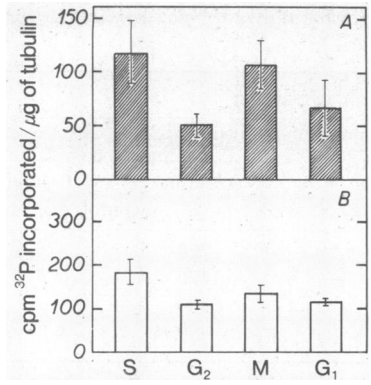

FIG. 4. Degree of phosphorylation of tubulin and phospholipids of the microtubular fractions isolated from HeLa cells at different stages in the cell cycle. Cells were grown through three generations in a phosphate-free complete medium supplemented with 1 μCi/ml of $^{32}P_i$ and were obtained at the indicated stages of the cell cycle as described in Table 1. The microtubules were isolated and the ^{32}P radioactivity incorporated into tubulin (A) and the bound phospholipids (B) were determined as described in detail under *Materials and Methods*. The results shown are the mean ± SEM of four independent experiments.

only in the protein fraction (tubulin), but also in the butanol-extractable fraction, which has been identified as phosphatidic acid (17). As can be observed in Table 1, the ^{32}P-incorporation into tubulin is maximal in M cells and minimal in G_2 cells*. No significant changes were observed in the incorporation of ^{32}P into the diglyceride component of the microtubular fraction.

These *in vitro* results suggest that changes in tubulin phosphorylation may occur during the cell cycle, and therefore evidence for such phenomenon *in vivo* was sought. For this purpose, HeLa cells were cultured in a phosphate-deficient medium containing $^{32}P_i$ for a period which began approximately two generations before synchronization and lasted until the cells were collected at the different stages of the cell cycle. This procedure insured that the intracellular pools, the tubulin, and the medium were labeled to a constant specific activity. The results shown in Fig. 4A indicate that the microtubular fractions obtained from cells in the S and M stages of the cycle contained twice as much protein bound phosphate as those arising from G_1 and G_2 cells.† It has been already shown (5) that the radioactivity incorporated into the protein fraction of HeLa microtubules migrates, upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, together with the tubulin band (molecular weight of 55,000), and it gives rise to [^{32}P]phosphoserine upon acid hydrolysis.

As already pointed out, we have recently observed that microtubules of different origin and prepared by independent methods have associated not only GTP and a protein kinase activity but also a diglyceride kinase and phospholipids (17). Hence, the $^{32}P_i$ incorporation into the microtubule-bound phospholipids was also determined, and it was found that it is enhanced only in the S phase (Fig. 4B).

Identical incorporations into tubulin and phospholipids (not shown) were obtained when griseofulvin (18 μg/ml) was substituted for colchicine in the preparation of G_2 and M cells by the synchronization Method II.

DISCUSSION

Tubulin phosphorylation is a process which has been now observed both *in vitro* and *in vivo* in several systems (1–5), but the functional significance of the covalent attachment of phosphate to tubulin is still unclear. Determination of the extent of phosphorylation of microtubules prepared from cells in different physiological situations might give useful information about the significance of this phenomenon. The variable phosphate content of tubulin observed in the microtubules isolated from various stages of the cell cycle of HeLa cells (Fig. 4) suggests that, indeed, the state of tubulin phosphorylation is altered in different cellular situations. The possibility that the observed changes in the degree of tubulin phosphorylation might be due to factors other than a phosphorylation–dephosphorylation process and its accompanying regulatory implications has to be considered. Some of these possibilities are that (*a*) the changing ratio of ^{32}P per protein (tubulin) during the cell cycle does not represent the actual phosphate content of tubulin. This is unlikely, since the cultures were grown through $2^1/_2$–3 generations in the presence of the label so that all pools were equilibrated to a constant specific activity. For instance, labeling of Chinese hamster cells with radioactive amino acids for $2^1/_2$ generations results in the total equilibration of the pools (18, 19). In addition, Karn *et al.* (16) have reported that the specific activity of the cellular ATP pool of synchronized HeLa cells is relatively uniform at the different stages of the cell cycle, even after a 15 min exposure to $^{32}P_i$. Hence, the ratio of covalently bound ^{32}P to tubulin determined in our experiments should reflect directly the phosphate content of tubulin. (*b*) The higher phosphate content of the M microtubules is due to some particular effect of colchicine, the substance used as blocking agent to increase the yield of mitotic cells (Method II). This is unlikely since griseofulvin—a drug which blocks cells also at metaphase but by an apparently different mechanism than colchicine (20)—gives identical results. Moreover, S microtubules also have a high ^{32}P/tubulin ratio, even though these cells have not been cultured in the presence of either drug. (*c*) There are two (or more) classes of tubulin in the cell which are not interconvertible, each one of them being *always* either phosphorylated or not, and their relative proportion changes during the cell cycle due to a different turnover. (*d*) Tubulin, recently synthesized, is unphosphorylated and, therefore, it is decreasing the P/tubulin ratio at some stages of the cell cycle. This possibility, and the previous one (*c*), seem also unlikely since the amount of tubulin present during the G_2 stage (3.7 μg/10^6 cells) is only about 20% higher than that found in S and G_1 phases, and 25–30% higher than in mitotic cells. Hence, the 2- to 3-fold lower phosphate content of the G_1 and G_2 microtubules, as compared to the S and M microtubules, cannot be accounted for by these fluctuations. Moreover, synthesis *de novo* of tubulin in synchronized HeLa cells is practically uniform through interphase (21).

* The maximal number of sites phosphorylated *in vitro* ranges between 0.06 and 0.59 mol of ^{32}P per mol of tubulin dimer (G_1 and M microtubules, respectively). Since M cells are only a small part of an asynchronous population, these figures are consistent with the 0.11 mol/mol value which can be calculated from the data of Fig. 2, and with the results obtained *in vitro* with chick embryonic muscle (5) and rat brain (4) tubulins.

† The final specific activity of the ^{32}P used in the *in vivo* experiments was not determined, and therefore no calculation of the number of moles of bound phosphate per tubulin molecule can be carried out.

The tubulin phosphorylation *in vivo* (Fig. 4) does not follow the same pattern as the microtubular protein kinase activity determined *in vitro* (Table 1, 3rd column) suggesting that, in addition to the amount of enzyme, other regulatory factors are probably acting. Moreover, the highest tubulin phosphorylation *in vitro* (Table 1, 4th column) does not occur at the cell stage in which phosphorylation *in vivo* is lowest (Fig. 4), as would have been expected had only one pool of microtubules been present and the *in vitro* phosphorylation taken place on those molecules which were not phosphorylated at the time of their isolation. Rather, the maximal *in vitro* phosphorylation of tubulin is parallel to the microtubular protein kinase activity (Fig. 3B and C and Table 1, 3rd–4th columns). The significance of these observations is not clear at the present time.

It is interesting to point out that the higher degree of HeLa tubulin phosphorylation is found in the S and M stages of the cell cycle, that is, at the time of active DNA synthesis and centriole replication (22) and of mitosis, respectively. Several reports (23–25) have described the presence of arms and bridges in the microtubules of the spindle apparatus of cultured human cells. The formation of this type of organelle might require not only the assembling of tubulin, but also its association to other proteins and/or cellular components, and this process might require a tubulin phosphorylation. The latter might be also required for the microtubule-mediated intracellular transport of certain components and/or the secretion of others. Mammalian brain tubulin can be polymerized *in vitro* (26–28), even in absence of added nucleotides (28); this suggests that a phosphorylation reaction *per se* is not necessary for tubulin aggregation. However, since the isolated tubulin already contains about 0.8 mol of phosphate per mol of dimer (2, 29), the possibility cannot be dismissed that the ability of tubulin to aggregate is dependent upon the presence of covalently bound phosphate. In this regard, Borisy and Olmsted (27) and Shelanski *et al.* (28) have reported that high-speed centrifugation of brain tubulin results in the loss of its capacity to polymerize *in vitro* to form microtubules. In addition, Kirschner *et al.* (30) have recently proposed that brain tubulin exists in two states, X- and Y-tubulin. The pellet and the supernatant obtained by the high-speed centrifugations (28) and the two fractions where the X- and Y-tubulins are present (30), are undistinguishable among each other and from pure tubulin by disc-gel electrophoresis. However, since some differences should exist to explain the different tubulin properties, the possibility that phosphate is involved should not be overlooked. More experimental work along these lines will be required before assessing the validity of these hypotheses.

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