aniline the regression equations obtained were as follows: $y = 0.988x + 0.695$, $r = 1.00$, $t = 0.358$, $P < 0.8 > 0.7$, $n = 12$; $y = 0.976x + 0.799$, $r = 1.00$, $t = 1.71$, $P < 0.2 > 0.1$, $n = 20$; and $y = 0.978x + 0.901$, $r = 1.00$, $t = 1.71$, $P < 0.1 > 0.05$, $n = 32$.

In the presence of 4-nitroaniline, values were $y = 0.986x + 2.50$, $r = 1.00$, $t = -2.0$, $P < 0.1 > 0.05$, $n = 12$; $y = 0.993x + 1.90$, $r = 1.00$, $t = -2.75$, $P < 0.02 > 0.01$, $n = 20$; and $y = 0.991x + 2.07$, $r = 1.00$, $t = -3.45$, $P < 0.005 > 0.001$, $n = 32$.

These results indicate that liberation of even 100 μmol of 4-nitroaniline per liter during the γ-glutamyltransferase assay would yield at most an average inhibition of 3% (cf. between-run precision of 2.05%) in a monitoring period of 5–10 min after the start of the reaction and that this inhibition would be absent in the 10–15 min monitoring period.

In view of the possibility that the (very minor) differences in the effect of 4-nitroaniline in the two 5-min monitoring periods might result from inhibition of the initial reaction rate by 4-nitroaniline, with a temporary lag-phase production, 18 of the above sera were re-examined with the instrument in the kinetic mode. In this mode, the absorbance change 2 min after serum addition is printed over three 15-s intervals. With each serum a linear print-out was obtained. A plot of the absorbance change over the total 45-s monitoring period in the presence of 4-nitroaniline (γ) vs. that obtained in its absence (x) yielded the following regression equation: $y = 0.961x + 3.01$, $r = 1.00$, $t = 2.01$, $P < 0.1 > 0.05$.

From the above data it is obvious that 4-nitroaniline, even at a concentration of 100 μmol/liter, produces minimal effect, even early in the course of the reaction, and that is no longer detectable at 10 min. With this 4-nitroaniline concentration, the substrate solution would have an absorbance of approximately 1.5 at 405 nm, compared with the usual absorbance of 0.5 for fresh substrate. Such a substrate would be visibly highly colored and normally would be rejected. For a 100 μmol/liter concentration of 4-nitroaniline to be released in the assay described in the second paragraph above would require serum with an activity of about 100 U/liter to act for 1 min. With the Abbott analyzer and a 51-fold serum dilution, it would require a serum of activity about 1000 U/liter acting over a 5-min period.

Because Martin et al. (2) have stated that 4-nitroaniline liberation even below 125 μmol/liter would lead to significant error with fixed-point assays, this point was re-investigated with such an assay (4), though I had originally investigated this possibility when developing the assay in 1968 and had found the reaction to obey zero-order kinetics for a 30-min period with (three) sera of activity up to 200 U/liter at 37 °C. This limit of linearity was clearly stated in the published method description. Reaction progress curves were again plotted, with Tris buffer and reagent concentrations as originally specified (4) but with a buffer pH of 8.3, and also with the identical technique but with AMPD buffer and reagent concentrations as above. The measuring instrument was the Vitatron photometer previously used in the kinetic studies. I again confirmed with two sera that the reaction progress curve was linear for a 30-min incubation period with activities up to 200 U/liter. With this assay, absorbance readings are made on a sixfold acid dilution of the assay mixture, and 30-min incubation of a serum of activity 200 U/liter liberates 4-nitroaniline at a final concentration of 600 μmol/liter of assay mixture.

It is clear that 4-nitroaniline liberation during either the kinetic or the fixed-point assays as above, produces such minimal inhibition, if any, that it may be ignored if normal precautions in enzyme measurement are observed, including the rejection of unsuitably colored substrate and monitoring for a time appropriate to the accepted linearity of the assay. The inhibition observed by Huseby and Strömme (1) may have resulted from the use of the LKB Reaction Rate Analyzer, in which the reaction rate is monitored within a few seconds of beginning the reaction; during this time, as shown above, there is the possibility that a high concentration of 4-nitroaniline already present might produce a (minor) lag phase in the reaction. Such inhibition might be methodologically significant within the context of the measuring procedure used by Huseby and Strömme where the reaction was "initiated" with glycylglycine and the authors were concerned of the possible effects of 4-nitroaniline liberation during the preincubation period of enzyme with substrate. However, even with the LKB Analyzer the reaction may be initiated with γ-glutamyl-p-nitroanilide substrate (3). A significant concentration of "pre-formed" 4-nitroaniline is then not present at the start of the reaction, and its liberation during the course of the reaction would not yield significant inhibition over a measuring period appropriate to the activity of the serum being measured. The suggestion of Martin et al. (2) that liberated 4-nitroaniline will inhibit the two-point assay appears to be without foundation, providing the stated limits of the assay are observed. With the authors’ two-point assay procedure the reaction is consistently linear for 30 min with sera of activity 200 U/liter at 37 °C, equivalent to a 4-nitroaniline concentration of 600 μmol/liter in the incubation medium at the termination of the reaction.

References

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Urinary Peptides Inhibit DNA Synthesis In Vitro in Certain Cultured Neoplastic Cells

To the Editor:

Normal human urine contains many different peptides, excreted in very small quantities (micrograms per liter) (1–9). Some of the peptides found in urine of patients seem to be characteristic for their disease (4–6). Many are biologically active. This was shown in a variety of systems. Especially, they may stimulate or inhibit normal and neoplastic growth processes. The best-known examples include kinins (7) and other peptides (8–11). The growth promoting and inhibiting properties may be conveniently studied in cells grown in cultures in vitro (12, 13).

In the experiments described previously, a technique was developed to separate urinary peptides soluble in ethanol/water (80/20 by vol) by use of

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free-flow electrophoresis into 17 fractions, which still are not homogeneous (1, 12), but which markedly differ in their biological activity on the growth of normal chicken fibroblasts (5) or various neoplastic cells (human leukemia, HeLa cells, human osteosarcoma (12)).

There were instances where the growth of neoplastic cells was inhibited more than that of normal cells (14, 15). This prompted us to perform trial experiments on the influence of some urinary peptides randomly available at the moment of the study, obtained from normal individuals, on the growth of a few cell lines of chemically induced neoplasms. The cell lines used included Morris and Reuber RH35 hepatomas, chemically induced in vivo in rats and maintained in vitro cultures for several years. The cells were grown in conditions described previously (16, 17), and in one instance the rat mammary adenocarcinoma M (18) was used. All were compared with normal rat liver or normal rat mammary cells cultivated in identical conditions, with and without the peptide. The results (Table 1) indicate that peptide fraction 6 is quite effective in inhibiting selectively the Reuber hepatoma RH35 DNA synthesis, as compared with the normal rat liver cells GRL.

Similarly, peptide fraction 2 inhibits DNA synthesis in this RH35 hepatoma more readily than in the normal cells. In contrast, the Morris hepatoma (HTC) was less sensitive to peptide fractions 3 and 9 than the normal GRL cells. Peptide fraction 5 did not influence DNA synthesis in either of those two cell lines (HTC and GRL). Similarly, fractions 3 and 8 did not affect DNA synthesis in normal mammary cells or those from mammary adenocarcinoma (SDA) to any significant degree.

The results presented extend the number of neoplastic cell lines selectively sensitive to various peptides (14) and encourage a systematic study of urinary peptides for their antineoplastic activity.

We thank Drs. G. M. Williams and P. C. Chan of the Naylor Dana Institute for Disease Prevention for providing us with the chemically induced neoplastic liver cells, and for the mammary adenocarcinomas. The study was aided by grants from the Muscular Dystrophy Association and the National Cancer Institute.

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Evaluation of a Commercially Available Colorimetric Method for Calcium as Performed with the Abbott ABA-100

To the Editor:

Recently, a colorimetric reagent for calcium has been made commercially available and proposed for use with the Abbott ABA-100 bichromatic analyzer, the Auto-Cal Reagent (Harleco, Gibbstown, N.J. 08027). We have evaluated this reagent by comparing results with it with those obtained by atomic absorption spectrometry. The reagent purports to offer advantages that include a smaller specimen volume, no precipitation of protein, shorter analysis time, and ease of use. We have compared the new reagent with the previously available colorimetric reagent (Abbott STA) and determined that at all dilutions examined the new reagent provides values that are significantly lower for all plasma samples examined (mean difference, 0.15 mg/100 ml; 95% confidence limits, 0.11-0.19 mg/100 ml; p < 0.001). The new reagent also provides higher values at all dilutions examined for various calcium-containing buffers (mean difference, 0.17 mg/100 ml) and for solutions containing calcium in deionized water (mean difference, 0.45 mg/100 ml).

We evaluated the new reagent at all dilutions examined in a variety of plasma samples obtained from normal subjects, patients with hypocalcemia, and patients with hypercalcemia. In each of these samples, the new reagent provided values that were significantly lower than those obtained with the STA reagent (mean difference, 0.14 mg/100 ml; 95% confidence limits, 0.10-0.19 mg/100 ml; p < 0.001). The new reagent also provided higher values for the same samples (mean difference, 0.17 mg/100 ml).

The new reagent was also evaluated for its ability to detect calcium levels in urine samples. The new reagent provided values that were significantly lower than those obtained with the STA reagent (mean difference, 0.20 mg/100 ml; 95% confidence limits, 0.15-0.24 mg/100 ml; p < 0.001). The new reagent also provided higher values for the same samples (mean difference, 0.45 mg/100 ml).

In summary, the new reagent for calcium appears to provide lower values than the STA reagent for all plasma and urine samples examined. The new reagent also provides higher values for all samples examined. The new reagent appears to be a promising new reagent for the determination of calcium.