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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF METABOLIC PRODUCTS FOR FERMENTATION CONTROL OF MAMMALIAN CELL CULTURE: ANALYSIS OF CARBOHYDRATES, ORGANIC ACIDS AND ORTHOPHOSPHATE USING REFRACTIVE INDEX AND ULTRAVIOLET DETECTORS

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## SUMMARY

A method for the determination of carbohydrate substrates and excreted metabolic end-products of cell culture supernatants using a strong cation-exchange column in the $H^+$ form has been developed. Organic acids and carbohydrates can be determined in addition to orthophosphoric acid. Pyrrolidone carboxylic acid, resulting from chemical conversion of the amino acid glutamine during the incubation of fresh medium and during the fermentation process, can be determined. The chromatographic method allows the correction of glutamine uptake values for physiological studies. Measured values of pyrrolidone carboxylic acid in supernatants of a human hybridoma cell line show that it cannot be consumed by the cells. This technique allows the separation of major metabolites used in process optimization. Peak homogeneity is proved by on-line monitoring of the effluent with an ultraviolet (214 nm) and a refractive index detector connected in series.

## INTRODUCTION

High-performance liquid chromatography (HPLC) has generally replaced the time-consuming enzymic or chemical analyses of single compounds. HPLC on a strong cation-exchange resin in the $H^+$ form allows the separation of carbohydrates in the presence of organic acids [1,2]. Owing to expanding applications of animal cell technology for the production of pharmaceutical compounds, the analytical control of such systems has become a necessity.

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Cell culture media are composed of a great number of different substances to obtain finely balanced nutrients [3,4]. Monitoring and control of metabolites is nowadays one of the optimization strategies [5–7] for cell culturists. The end-products of glutamine and glucose catabolism, e.g. ammonium, lactate and carbon dioxide, are excreted into the medium. These end-products may inhibit cell growth and product formation. Lactate has been shown to inhibit cell growth of hybridoma cells [8,9]. Glutamine is not stable under fermentation conditions and forms pyrrolidone carboxylic acid (pyroglutamine, pyroglutamic acid) and ammonium. This non-enzymic cyclization of glutamine is dependent on pH and temperature [10] and has to be taken into account in calculations of metabolic consumption rates. Many authors use standard decay rates for glutamine or even neglect this effect. There is evidence that even the amount of fetal calf serum, added to the media to supply cells with growth factors, influences the cyclization of glutamine [11].

## EXPERIMENTAL

### *Apparatus*

The chromatographic system consisted of an HPLC pump (Model 114M, Beckman Instruments, Berkeley, CA, U.S.A.), a refractive index detector (Model 156, Beckman), a UV detector (Model 163, Beckman) and an LKB 2153 autoinjector with a 20-$\mu$l loop (LKB, Bromma, Sweden). The column was an Aminex HPX87H (300 mm$\times$7.8 mm I.D., Bio-Rad, Richmond, CA, U.S.A.) with a cation-$H^+$ guard column cartridge (30 mm$\times$6 mm I.D., Bio-Rad). Data acquisition for two signals and integration was performed on an IBM compatible computer (Aquarius Systems International, Taipeh, Taiwan) using Nelson Chromatography software Version 4.0 (Nelson Analytical, Cuppertino, CA, U.S.A.)

### *Solvent*

Sulphuric acid (0.005 *M*) was prepared by diluting a 0.5 *M* solution obtained from E. Merck (Darmstad, F.R.G.) to the appropriate volume with doubly distilled water.

All reference standard solutions were prepared from analytical reagent grade substances obtained from Sigma (St. Louis, MO, U.S.A.). Ammonia was analysed after protein precipitation with an equal volume of 5% sulphosalicylic acid using a Wescan Model 360 continuous flow ammonia analyser.

### *Sample preparation*

Cell culture samples were taken from the fermentor and centrifuged for 10 min at 220 *g*. The supernatant was filtered through a 0.2-$\mu$m filter (Millex GS, Millipore, Molsheim, France) prior to injection.

## RESULTS AND DISCUSSION

Orthophosphate is one of the essential compounds for living organisms and is always present in biological material. Chromatographic resolution had to be optimized for the separation of the orthophosphoric acid (phosphate)–citric acid pair, which can be released by cells. The temperature dependence of the $k'$ values is shown in Fig. 1 for this pair and for glucose. Because of the hydrolising effect of the solvent at higher temperatures for disaccharides and of possible degradation of other compounds, such as glutamine, the temperature was adjusted to 25 °C. In order to avoid higher back-pressures at low temperatures the flow-rate was adjusted to 0.45 ml/min. Multi-level calibration at six concentration levels, ranging from 40 $\mu M$ to 11 m$M$, was used for calibration. Correlation coefficients varied between 0.999 and 0.995. Chromatograms of a standard solution, of a freshly prepared medium and of a cell culture supernatant are shown in Figs. 2, 3 and 4, respectively.

Using this type of column for biological samples such as cell culture supernatants, it is necessary to combine the refractive index detector with a UV detector set at 214 nm to evaluate peak purity by taking the ratio of the peak areas of the two detection systems: the factor calculated from the resulting slope of the calibration curve for UV detection divided by that of the RI detection is constant for a given substance under given chromatographic conditions.

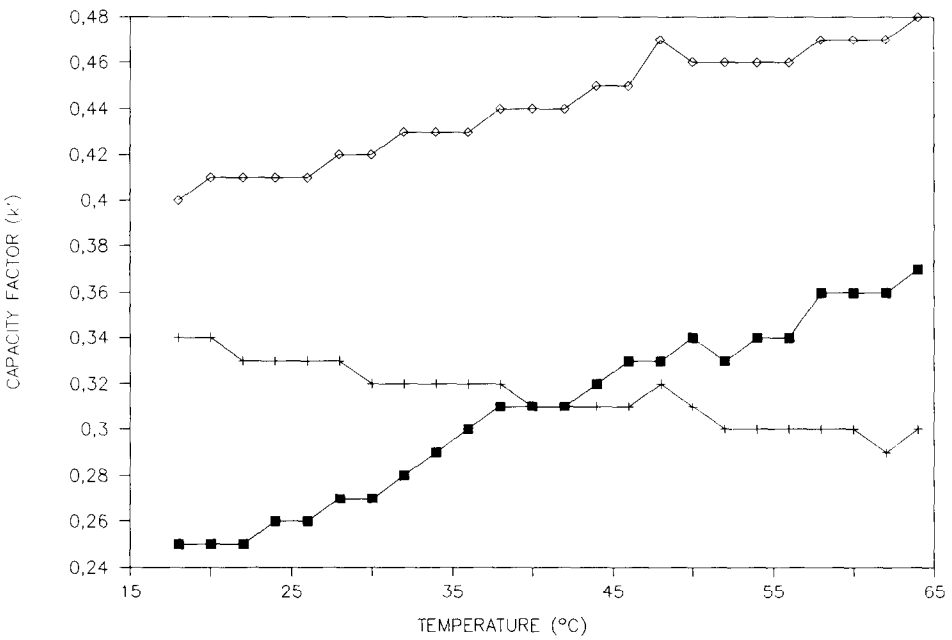


Fig. 1. $k'$ values for phosphoric acid (■), citric acid (+) and glucose (◇) versus temperature. Conditions: column, HPX87H cartridge system; mobile phase, 0.005 $M$ sulphuric acid; flow-rate, 0.45 ml/min.

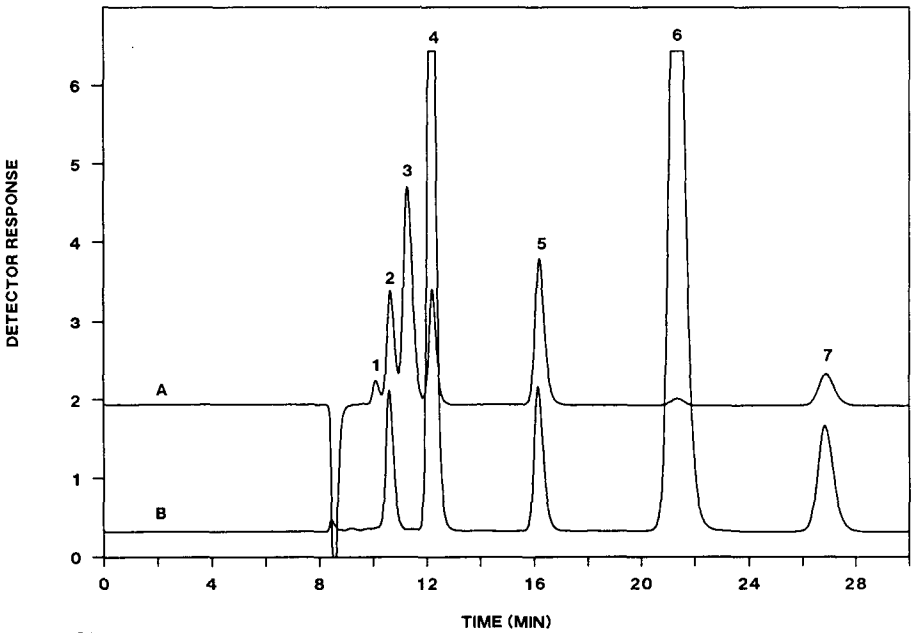


Fig. 2. Chromatogram of a standard solution. Detection: (A) refractive index (30 mV full scale); (B) UV 214 nm (300 mV full scale). Peaks: 1 = phosphoric acid (21 nmol); 2 = citric acid (52.0 nmol); 3 = glucose (111 nmol); 4 = pyruvic acid (114 nmol); 5 = lactic acid (222 nmol); 6 = fumaric acid (8.6 nmol); 7 = pyroglutamic acid (38.8 nmol). Column temperature, 25 °C; other conditions as in Fig. 1.

TABLE I

QUOTIENTS OF RESPONSE FACTORS (UV DETECTION AT 214 nm/RI DETECTION) OF CARBOXYLIC COMPOUNDS

Chromatographic conditions as in Fig. 2.

| Compound | Quotient | Compound | Quotient |
|---|---|---|---|
| Acetaldehyde | 0.1 | Isocitric acid | 5.4 |
| Glyceraldehyde | 0.7 | Lactic acid | 6.1 |
| Fructose | 0.8 | Malonic acid | 6.5 |
| Dihydroxyacetone | 1.5 | Citric acid | 7.3 |
| Galacturonic acid | 2.1 | Acetoacetic acid | 7.8 |
| Gluconic acid | 3.0 | Formic acid | 13.0 |
| Galactonic acid | 3.4 | N-Acetylglycine | 16.0 |
| Succinic acid | 3.8 | $\alpha$-Ketoglutaric acid | 18.8 |
| Glyceric acid | 5.0 | Pyruvic acid | 53.8 |
| $\alpha$-Hydroxybutyric acid | 5.3 | Fumaric acid | 565 |
| | | *cis*-Aconitic acid | 566 |

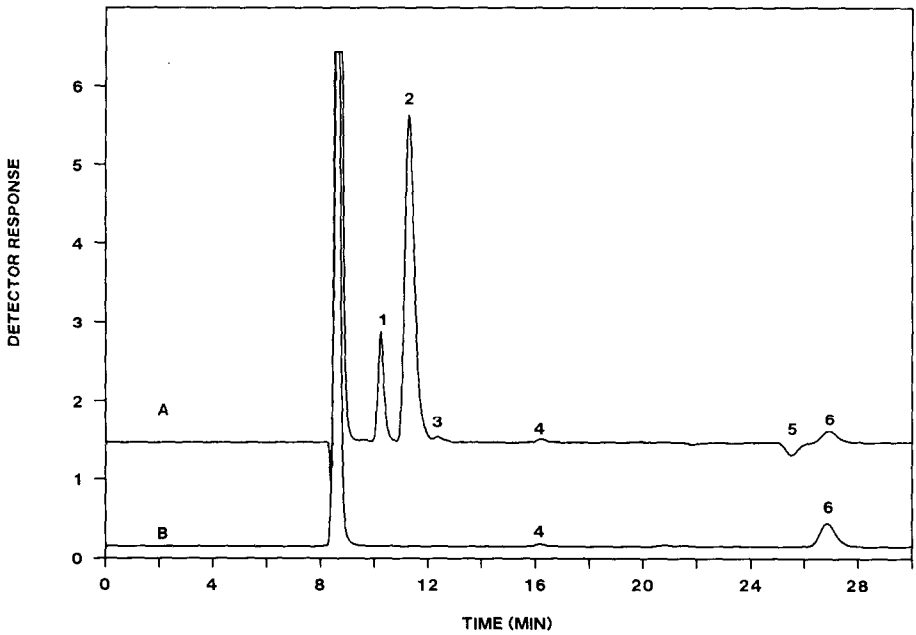


Fig. 3. Chromatogram of fresh culture media RPMI 1640 (Biochrom, G.D.R.) + 2% fetal calf serum (Biological Industries, Israel). Conditions as in Fig. 1; column temperature, 25 °C. Detection: (A) refractive index (30 mV full scale); (B) UV 214 nm (300 mV full scale). Peaks: 1 = phosphoric acid; 2 = glucose; 3 = myoinositol; 4 = lactic acid; 5 = carbonic acid; 6 = pyroglutamic acid.

The factors for different types of carboxylic compounds are given in Table I. The response of the UV detector at 214 nm is up to 600 times higher (unsaturated C–C bonds) than that of the refractive index detector for different kinds of organic compounds. Besides retention times, these factors are a second qualitative parameter for substance characterization.

Owing to the release of other substances during cell lysis, peak purity must be controlled. Under well perfused cell culture conditions no interferences with other compounds were detectable. During cell lysis the resulting chromatograms from the refractive index detector were unchanged, because the concentrations did not reach the lower limit of detection, but the more sensitive UV detector showed additional peaks. Retention times of some substances of biological interest are given in Table II.

Using the Nelson analytical software, data from two channels can be collected at the same time and calculated by different methods. Two independent calculations for one substance can be performed and lead to equal results when pure peaks are eluted from the column. This combination of two detection systems allows rapid control of peak purity. It should be mentioned that calculation of concentrations of two known substances that are not resolved and one of which absorbs in the UV is possible. An example of such an unresolved

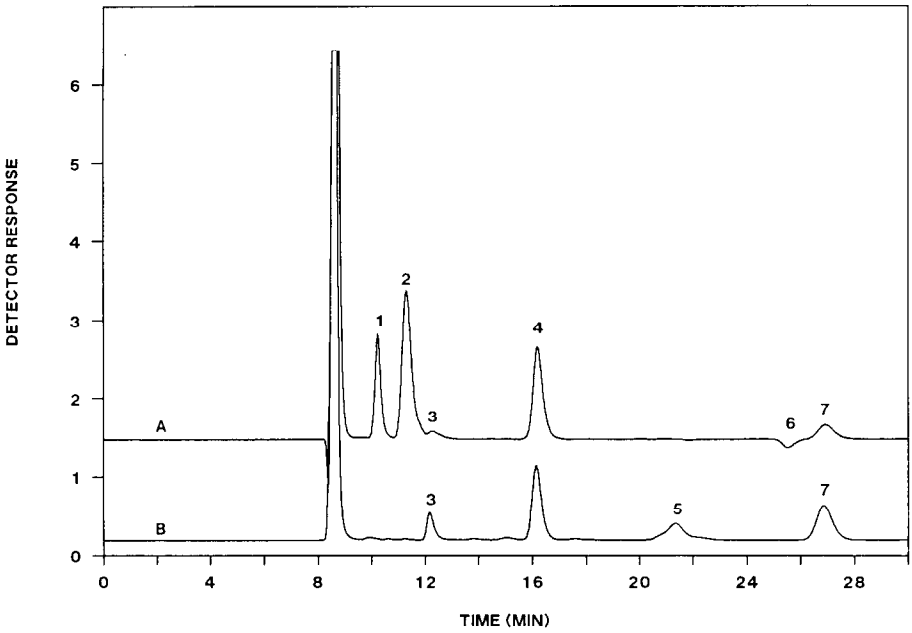


Fig. 4. Chromatogram of the cell supernatant. Conditions as in Fig. 1; column temperature, 25 °C. Detection: (A) refractive index (30 mV full scale); (B) UV 214 nm (300 mV full scale). Peaks: 1 = phosphoric acid; 2 = glucose; 3 = pyruvic acid; 4 = lactic acid; 5 = fumaric acid; 6 = carbonic acid; 7 = pyroglutamic acid.

pair is glucose and $\alpha$-ketoglutaric acid. The mean values of eight analyses of a standard mixture consisting of 11.11 m*M* glucose and 13.39 m*M* $\alpha$-ketoglutaric acid were detected with the refractive index detector and calculated as glucose 21.42 m*M* (S.D., 0.21 m*M* = 0.98%) and 13.61 m*M* (S.D., 0.17 m*M* = 1.26%) with UV detection for $\alpha$-ketoglutaric acid. The corrected value for glucose was 11.17 m*M* (S.D., 0.21 m*M* = 1.84%).

TABLE II

RETENTION TIMES (UV DETECTION 214 nm) OF OTHER ORGANIC ACIDS THAT MIGHT APPEAR DURING FERMENTATION

| Retention time (min) | Substance | Retention time (min) | Substance |
|---|---|---|---|
| 9.80 | *cis*-Aconitic acid | 14.48 | Glyceric acid |
| 10.29 | Oxalacetic acid | 14.72 | *trans*-Aconitic acid |
| 10.98 | Isocitric acid | 16.65 | Succinic acid |
| 11.16 | Galacturonic acid | 18.95 | Formic acid |
| 11.35 | Maleic acid | 20.41 | Acetic acid |
| 12.32 | Galactonic acid | 24.89 | N-Acetylglycine |
| 13.45 | Malonic acid | 27.46 | Butyric acid |

The isocratic elution system is stable under the above conditions. Baseline noise is of the order of 22 $\mu$V for the refractive index and 19 $\mu$V ($\mu$AU) for the UV detector (both 1 V full scale). Column lifetime is greater than 4000 analyses, although the guard column has to be changed after 950 analyses. Table III shows the precision of the assay calculated from eighteen runs of standard samples detected using both detectors. The UV detection system affords higher sensitivity and reproducibility for organic acids, but the universal refractive index detector allows the ready detection of the orthophosphoric acid and carbohydrates without the need for derivatization.

Tables IV and V show the results obtained with the two detectors for a cell culture supernatant and a 1:1 mixture of the sample with the standard solution (see Table III). A signal-to-noise ratio of 2:1 is achieved at amounts of 170 pmol orthophosphoric acid injected on column.

TABLE III

RELATIVE STANDARD DEVIATIONS OF PEAK AREAS USING TWO DETECTION SYSTEMS CONNECTED IN SERIES

| Substance | Concentration (m*M*) | Relative standard deviation ($n=18$) (C.V., %) | |
|---|---|---|---|
| | | RI detector | UV detector |
| Orthophosphoric acid | 1.05 | 2.33 | – |
| Citric acid | 2.60 | 0.85 | 0.70 |
| Glucose | 5.55 | 0.94 | – |
| Pyruvic acid | 5.68 | 1.04 | 0.84 |
| Lactic acid | 11.10 | 1.03 | 0.56 |
| Fumaric acid | 0.43 | 4.10 | 0.65 |
| Pyroglutamic acid | 1.94 | 2.95 | 0.71 |

TABLE IV

MEAN VALUES AND RELATIVE STANDARD DEVIATIONS OF CONCENTRATIONS FROM FIFTEEN ASSAYS OF A CELL CULTURE SUPERNATANT USING TWO DETECTION SYSTEMS CONNECTED IN SERIES

| Substance | RI detector | | UV detector | |
|---|---|---|---|---|
| | Concentration (m*M*) | C.V. (%) | Concentration (m*M*) | C.V. (%) |
| Orthophosphoric acid | 6.27 | 1.43 | – | |
| Citric acid | Not detected | | 0.02 | 2.34 |
| Glucose | 9.79 | 0.76 | – | |
| Pyruvic acid | 0.05 | 6.22 | 0.03 | 2.18 |
| Lactic acid | 4.92 | 1.24 | 4.97 | 0.48 |
| Fumaric acid | Not detected | | 0.008 | 3.48 |
| Pyroglutamic acid | 0.17 | 5.35 | 0.15 | 2.16 |

TABLE V

MEAN VALUES AND RELATIVE STANDARD DEVIATIONS OF CONCENTRATIONS FROM FIFTEEN ASSAYS OF SPIKED CELL CULTURE SUPERNATANT USING TWO DETECTION SYSTEMS CONNECTED IN SERIES

| Substance | RI detector | | UV detector | |
|---|---|---|---|---|
| | Concentration (m*M*) | C.V. (%) | Concentration (m*M*) | C.V. (%) |
| Orthophosphoric acid | 3.64 | 2.01 | – | |
| Citric acid | 1.31 | 1.97 | 1.27 | 1.56 |
| Glucose | 7.65 | 0.93 | – | |
| Pyruvic acid | 2.86 | 1.49 | 2.85 | 1.07 |
| Lactic acid | 8.05 | 1.29 | 7.99 | 0.66 |
| Fumaric acid | 0.26 | 6.33 | 0.21 | 0.92 |
| Pyroglutamic acid | 1.07 | 4.01 | 1.06 | 1.14 |

The method described allows the off-line determination of pyroglutamine, which results from chemical conversion of glutamine during cell culture processes. Besides glucose, glutamine is the major source of energy and the amino nitrogen for many types of cell lines; up to 30% of the energy requirements of human diploid fibroblasts can be provided by glutamine in the conventional medium with serum [12]. For process optimization of cell cultures, uptake

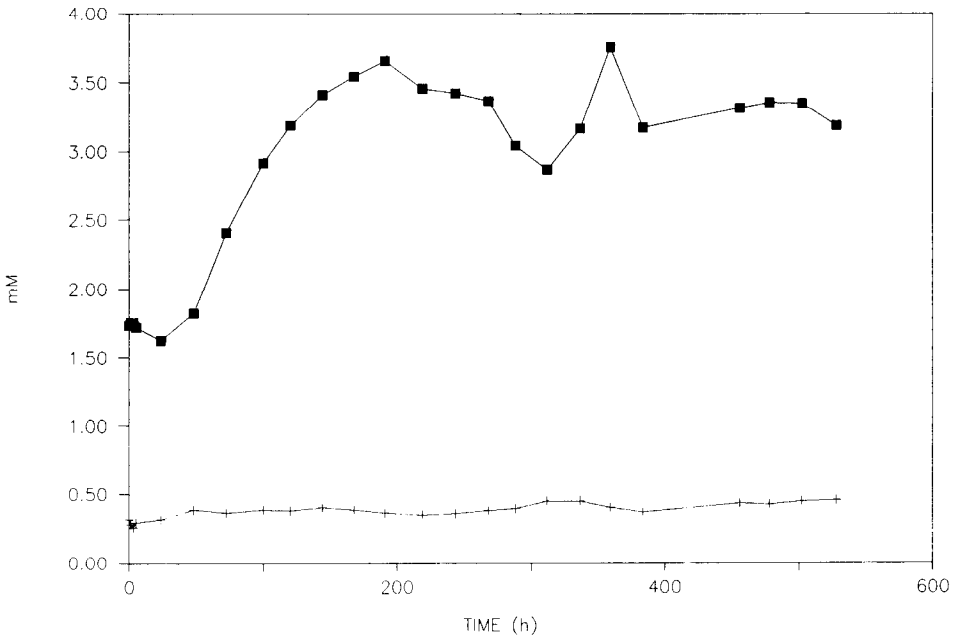


Fig. 5. Total ammonia (■) and values of pyroglutamic acid (+) during a continuous culture fermentation of mammalian cells.

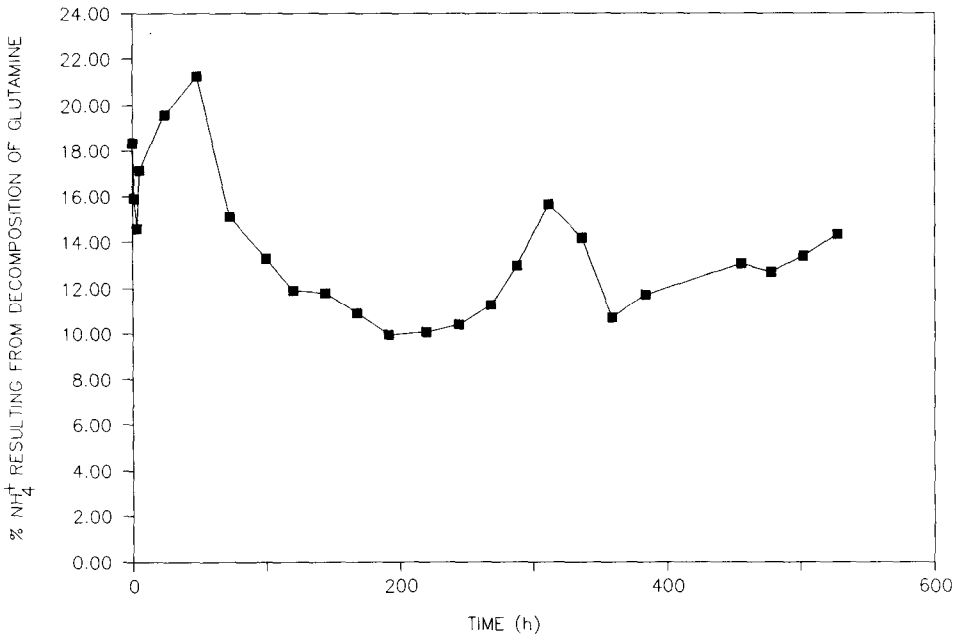


Fig. 6. Percentage of ammonia in culture supernatants due to decomposition of glutamine.

rates of amino acids are calculated and the medium composition is changed [5–7]. The determination of pyroglutamine and ammonia during fermentation of human hybridomas shows that, on average, 14% of the total ammonia concentration results from chemical decomposition of glutamine (medium concentration 4 m*M*) (Figs. 5 and 6). A differentiation between ammonia derived chemically and that produced by cell activity is possible. These values depend on the amount of glutamine and on culture conditions. In batch culture, up to 45% of glutamine is converted into pyroglutamine and ammonia (data not shown). Data obtained by HPLC show that human and mouse hybridoma cell lines do not consume pyroglutamic acid, even under glutamine and glutamate limiting conditions.

This chromatographic method offers a suitable control system for physiological parameters of cell culture systems. For the calculation of glutamine consumption and ammonia production the described determination of pyroglutamic acid is essential.

REFERENCES

1 G. Bonn, R. Pecina, E. Burtscher and O. Bobleter, J. Chromatogr., 287 (1984) 215.
2 G. Bonn, J. Chromatogr., 322 (1985) 411.
3 H. Eagle, Science, 130 (1959) 432.

4 G.E. Moore, R.E. Gerner and H.A. Franklin, J.Am.Med.Assoc., 199 (1967) 519.
5 M.W. Glacken, Biotechnology, 6 (1988) 1041.
6 W.M. Miller, C.R. Wilke and H.W. Blanch, Bioprocess Eng., 3 (1988) 113.
7 N.G. Ray, S.B. Karkare and P.W. Runstadler, Jr., Biotechnol. Bioeng., 33 (1989) 724.
8 S. Reuveny, Dev. Biol. Stand., 66 (1987) 196.
9 H.R. Zielke, C.M. Sumbilla, D.A. Sevdalian, R.L. Hawkins and P.T. Ozand, J. Cell. Phys., 104 (1989) 433.
10 M. Orlowski and A. Meister, in P.D. Boyer (Editor), The Enzymes, Vol. IV, Academic Press, New York, 1971, p. 123.
11 A. Lin and P. Agrawal, Biotechnol. Lett., 10 (1988) 695.
12 K.J. Lambert and J.R. Birch, in R.E. Spier and J.B. Griffiths (Editors), Animal Cell Biotechnology, Vol. I, Academic Press, New York, 1985, p. 85.