Quantitative Determination of Amino Acids in the 10^{-14} Molar Range by Scanning Microscope Photometry[1]

1-Di-methyl-Amino-Naphthalene-5-Sulfonyl-chloride (Dansyl-chloride) reacts with the amino or hydroxyl groups of free amino acids[2,3]. The resultant acid stable compound is called a dansylated amino acid. This compound fluoresces, and this property is used to measure free amino acids in the nanomole range[2]. The dansylated amino acids are separated by thin layer chromatography on polyamide layers (15 × 15 cm) and identified by their fluorescence at characteristic positions on the chromatogram. A further increase in sensitivity down to the picomole range can be achieved by smaller polyamide layers (3 × 3 cm)[4].

The use of C^{14}-labelled Dansyl-chloride allows a quantitative analysis of free amino acids on the same scale. After identification of the fluorescent dansylated amino acids on the polyamide layer (3 × 3 cm) the spots are transfered into vials for measuring their radioactivity in a liquid scintillation counter[5,6]. The sensitivity of this method is limited by the fluorescence of the dansylated amino acids. If the fluorescence becomes so small that the spot cannot be detected anymore after microchromatography, the maximal sensitivity of this method is reached.

In order to measure total amounts of amino acids lower than the picomole range the method has been further modified by putting the polyamide layer with the C^{14}-labelled amino acids on a photographic plate. After microautoradiography the density of the spots on the film was

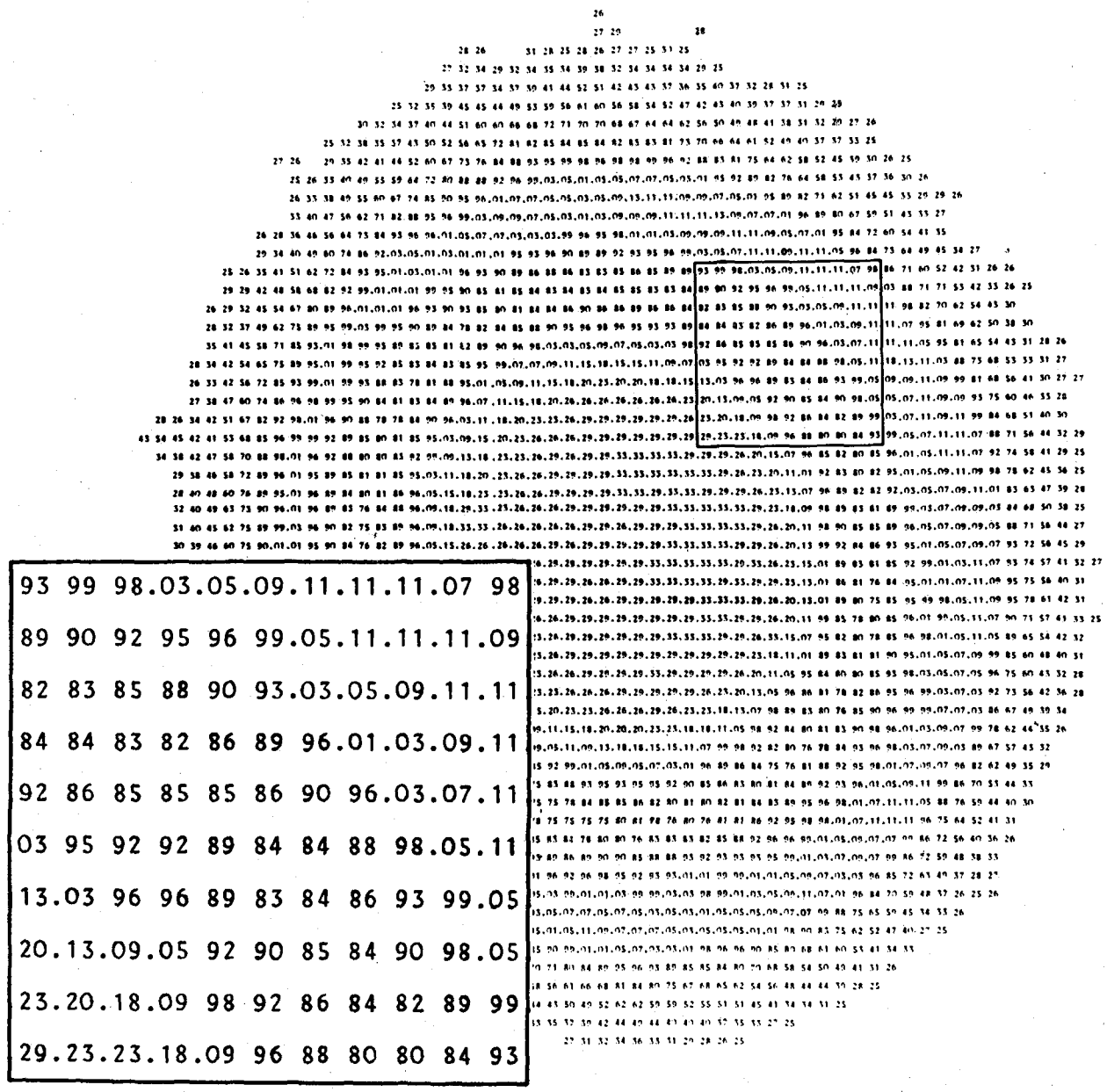

Fig. 1. Single spot extinction values of an autoradiogram (number of single values $n = 2000$). For example 86 means an extinction of *0.86 and 0.03* means 1.03 (For definition of extinction see manual of CYTOSCAN, Carl Zeiss, Oberkochen, W-Germany). The total extinction of this spot is 208736. The magnification of the section is × 3.

measured by scanning microscope photometry. With this method amino acids could be determined quantitatively in the range of 10^{-14} moles.

Methods. Dansylation and microchromatography: A 60 nl rat urine sample is transfered with a thin capillary under a Zeiss stereo microscope into a 2 µl capillary tube (i.d. 0.28 mm, length 32 mm, Drummond microcap) sealed at the bottom. After adding 60 nl of 0.2 molar $NaHCO_3$ and 120 nl C^{14}-Dansyl-chloride in acetone (2 mg/ml; 8.3 mCi/m*M*; CEA, Saclay, Gif-sur-Yvette, France) the top of the microcap is sealed with a small flame. Mixing of the sample is done by centrifuging the solution in a low speed table centrifuge 3 times from one end to the other end of the closed microcap. After incubation for 1 h at 37 °C in the dark the sample is transfered with a thin capillary to one corner of a 3×3 cm polyamide layer (Schleicher and Schuell, Einbeck, W-Germany) under a stereo microscope. After drying the layer with a hair dryer, chromatography is started in 3% formic acid in the first dimension. After drying, chromatography in the second dimension is done in benzene: glacial acetic acid (9:1, v/v).

Equal amounts of C^{14}-Dansyl-chloride standard solutions diluted 1:100, 1:1000, and 1:10,000 are transfered quantitatively onto the chromatogram for a calibration curve.

Microautoradiography: High sensitive radiation dosage meter films (Agfa Gevaert, Leverkusen, W-Germany) are used for microautoradiography. This film is laid on the radioactive chromatogram and fixed with 2 slides on both sides. The film is exposed for 108 h at room temperature, processed for 3 min with Gevaert developer, rinsed with water, put in fixative for 3 min and dryed by air.

Scanning microscope photometry: The single dark spots on the autoradiograms are quantitatively measured by the Zeiss scanning microscope photometer[7]. In all spots the intensity is distributed inhomogenously. There is much variabilty in size and shape of the spots. The use of the scanning microscope photometer allows an accurate measurement of the area and intensity. The spots are scanned, the intensities and areas are measured by extinction from an image-converter and the data are put into a computer. This was done with a magnification of 5 times, a wave length of 546 nm, a step size of 50 µm and a measurement diaphragm of 0.5 mm.

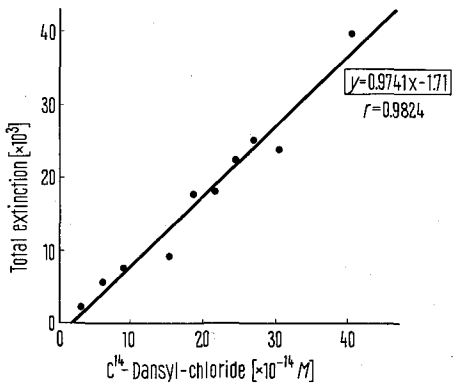

Fig. 2. The graph shows the regression line between the amount of C^{14}-Dansyl-chloride from 1 to 40×10^{-14} *M* on the abscissa and the total extinction of the spot on the autoradiogram, on the ordinate.

[1] Supported by the Deutsche Forschungsgemeinschaft.

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[7] We would like to acknowledge the cooperation of Mr. HUB, Mr. WINTERSTEINER and Dr. ZIMMER, Fa. Zeiss, Oberkochen, W-Germany.

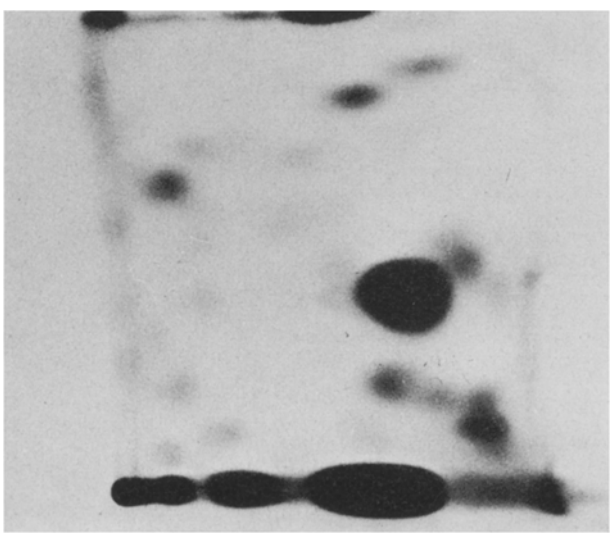

Fig. 3. Photograph of an original autoradiogram showing the C^{14}-labelled Dansyl-amino acids of a 60 nl rat urine sample. The starting point is in the left lower corner. The dark spot in the right middle of the autoradiogram is Dansyl-NH_2. The other less intensitive spots represent different Dansyl-amino acids.

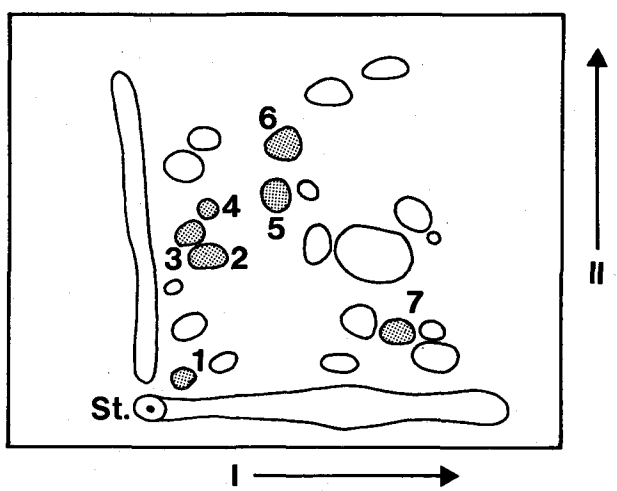

Fig. 4. Copy of the autoradiogram of Figure 3. St, starting point; I, first dimension of chromatography (3% formic acid); II, second dimension of chromatography (90% benzene, 10% glacial acetic acid, v/v) 1, Tryptophane; 2, Phenylalanine; 3, Histidine; 4, Leucine; 5, Valine; 6, Proline; 7, Hydroxyproline.

Table I. Measured values of total extinction of standard C^{14}-Dansyl-chloride samples on the microautoradiogram

Amount of C^{14}-Dansyl chloride (M)	Total extinction
3×10^{-14}	2,300
6×10^{-14}	5,600
9×10^{-14}	7,400
15×10^{-14}	9,300
18×10^{-14}	17,000
21×10^{-14}	17,400
24×10^{-14}	22,600
27×10^{-14}	25,300
30×10^{-14}	24,000
40×10^{-14}	40,000

Tabelle II. Measured values and calculated amounts from the graph (Figure 2) of 7 amino acids in 60 nl of rat urine

Amino acid	Total extinction	Amount of amino acid (M)
1. Tryptophane	18,690	20×10^{-14}
2. Phenylalanine	10,668	$12{,}75\times10^{-14}$
3. Histidine	3,540	2.7×10^{-14} (5.4×10^{-14})
4. Leucine	7,350	9.3×10^{-14}
5. Valine	8,375	10.3×10^{-14}
6. Proline	14,433	14.5×10^{-14}
7. Hydroxyproline	27,900	30.4×10^{-14}

When there is a complete binding of amino acids with C^{14}-Dansyl chloride under these conditions, the molar ratio of amino acid to C^{14}-Dansyl chloride is one to one. (In the case of Histidine this ratio is 1:2. Histidine binds 2 Dansyl-molecules).

Figure 1 shows the measured intensities of one spot. The computer calculates the mean intensity of the total spot and the total area. The multiplication of total area by mean intensity gives the total intensity of the spot (cf. Table I). The background and extinction mainly depend on the sensitivity of the film, the time of exposure and the conditions of developing and fixation. The same background value is used for all spots on one autoradiogram. The diameter of the spot should not be more than 1 cm. Spots which are too close cannot be separated. When using higher amounts of radioactivity under these conditions, the calibration curve is no longer linear and reaches a maximum point of total intensity.

Results and discussion. Table I gives the measured data for total intensity of several C^{14}-Dansyl-chloride samples with a known concentration which have been transfered directly to the polyamide layer and subsequently autoradiographed. There is a linear correlation between total intensity and concentration ($r = 0.9842$, Figure 2). This good correlation is proof that the described technique can be applied to very small amounts of biological material. With higher radioactivities this linear relationship would not be found anymore and therefore a shorter exposure of the film would be necessary.

An autoradiogram of C^{14}-dansylated amino acids in 60 nl of rat urine was done. Single spots representing certain amino acids were measured with this new technique (Figures 3 and 4).

Table II gives the measured total intensity and the calculated concentration of 7 amino acids. It can be seen that these amino acids have a concentration in the range of 10^{-14} molar.

Zusammenfassung. Die beschriebene Methode stellt eine Kombination der Mikrochromatographie und der «Scanning-Mikroskop-Photometrie» dar und ermöglicht eine quantitative Bestimmung von C^{14}-markierten Dansyl-Aminosäuren in biologischem Material im Bereich von $10^{-14}M$.

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Direct Measurement of Interphase Shortening Produced by Kinetin plus Indol Acetic Acid in Meristematic Cells of *Allium cepa* L.

There are a number of papers dealing with different aspects of the effect of kinetin or kinetin plus indol acetic acid (IAA) on plant cells[1–4], but in the field of its action on the duration of the cell division cycle there is some confusion due to several contradictory reports.

The experiments here described intend to show, by using a synchronous binucleate cell population induced by caffeine in root meristems, whether these growth factors, at certain concentration, are able to shorten the duration of the cell division cycle.

The material used was the root meristem of *Allium cepa* L. The onion bulbs were grown in the dark at the constant temperature of $15° \pm 0.5$ °C in cylindrical receptacles of 70 cm^3 capacity, with tap water which was renewed every 24 h and aerated continuously by bubbling in 10–20 cm^3 of air/min. The bulbs were so placed that only their bases remained submerged in the water.

The roots were fixed in a 3:1 mixture of ethanol-acetic acid and the specimens were prepared by staining the roots with acetic orcein, according to the technique of Tjio and Levan[5].

Labelling with caffeine. The roots, attached to the bulbs, were submerged in 0.1% caffeine for 1 h. This drug inhibits cytokinesis in cells going through the telophase during this time and produces a binucleate population

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