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DETERMINATION OF THE SPECIFIC RADIOACTIVITY OF AMINO ACIDS BY A COMBINATION OF THIN-LAYER CHROMATOGRAPHY AND QUANTITATIVE AUTORADIOGRAPHY

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SUMMARY

A new method for the analysis of the specific activity of amino acids is described. The analysis is carried out by thin-layer chromatography of the dansylated amino acids, computerized fluorescence evaluation and activity measurement by quantitative autoradiography. Quantitative evaluation of the autoradiographs is achieved by careful calibration of the X-ray film blackening. As shown for ^{14}C-labelled phenylalanine and tyrosine, the method allows the simultaneous determination of the specific activity of 22 amino acids. About 10^{-13} mol of an amino acid with a specific activity of less than 5 GBq/mmol can be detected and measured by this method.

INTRODUCTION

In principle, the determination of the specific activity of a mixture of labelled amino acids is a three-step procedure: (1) separation of the mixture into the individual amino acids; (2) determination of the respective amino acid concentrations; (3) activity measurement of the amino acids.

The reaction of amino acids with 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride) and subsequent separation of the dansyl derivatives by two-dimensional thin-layer chromatography (TLC) has been shown to be a valuable tool for amino acid separation[1–3]. This method, described in detail by Neuhoff[1], allows the measurement of amino acid concentrations of about 10^{-13} mol/l in nanolitres of the sample[4]. Computer-controlled fluorescence evaluation of a single chromatogram enables the simultaneous determination of the concentration of more than 22 amino acids and related compounds[5,6].

Up to now, for activity measurement it was necessary to scrape off the separated amino acids together with the carrier-sheet and measure the respective activity by liquid scintillation counting (LSC). This method is very time consuming and laborious and, particularly when applied to the micro scale, involves several sources of error, *e.g.*, the possible loss of material during the excising procedure.

In this paper we report a method which avoids these disadvantages by the quantitative evaluation of autoradiographs obtained from the respective chromatograms. Although described in detail here only for [14C]phenylalanine and [14C]tyrosine, the procedure allows the simultaneous measurement of the specific activity of more than 22 amino acids and related compounds by computer-controlled evaluation of a single chromatogram and a single autoradiograph.

MATERIALS AND METHODS

Materials

All the radioactive chemicals were obtained from Amersham Buchler (Braunschweig, F.R.G.) and their specific activities are given in the text. Amino acids (p.a.) were obtained from Sigma (St. Louis, MO, U.S.A.), dansyl chloride from Serva (Heidelberg, F.R.G.). All the other chemicals (p.a.) were from Merck (Darmstadt, F.R.G.). The TLC-Micropolyamide foils were obtained from Schleicher & Schüll (Dassel, F.R.G.).

Separation of the amino acids

The amino acids were separated by micro TLC of the dansylated sample according to Neuhoff[1], using the procedure described by Schott et al.[7]; $2 \cdot 10^{-4}$ mol/l
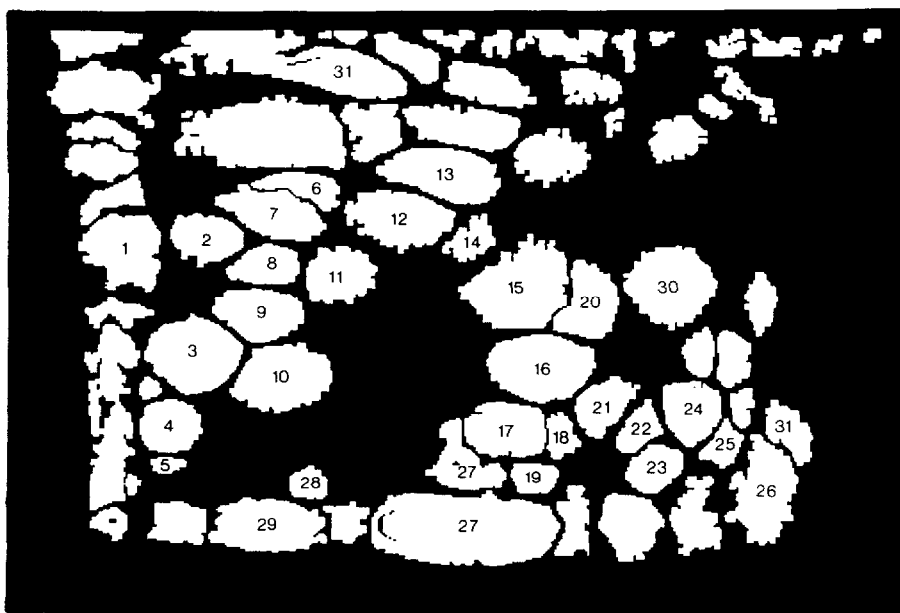

Fig. 1. Computer plot of the position of 22 amino acids and related compounds after separation of their dansyl derivatives on micro thin-layer chromatograms. Substances: 1 = bis(dansyl)tyrosine; 2 = His; 3 = Lys; 4 = Trp; 5 = dansyl-N-serotonin; 6 = Ile; 7 = Leu; 8 = Phe; 9 = α-phenylglycine (standard); 10 = Orn; 11 = Met; 12 = Val; 13 = Pro; 14 = γ-aminobutyric acid (GABA); 15 = Ala; 16 = Gly; 17 = Glu; 18 = m-hydroxy-Tyr; 19 = Asp; 20 = ammonia; 21 = hydroxyproline; 22 = Thr; 23 = Ser; 24 = Gln; 25 = Asn; 26 = Arg; 27 = dansyl hydroxide; 28 = p-hydroxy-Tyr; 29 = Tau; 30 = Eta; 31 = dansyldiethylamine. The separation procedure is described in Materials and methods.

D-α-phenyl[1-^{14}C]glycine (specific activity 1.78 TBq/mol, 81.4 kBq/ml) were added as an internal standard. The amino acids were treated with dansyl chloride and subsequently separated by two-dimensional TLC on 3 cm $\times$ 4 cm Micropolyamide sheets. An example of the location of the dansyl amino acid spots on a chromatogram is given in Fig. 1.

Determination of the amino acid concentration

The yield of the dansyl reaction and hence the fluorescence intensity of the chromatographic spots varies for each single amino acid as a function of the pH, temperature and composition of the amino acid pool[3]. For this reason the relative fluorescence intensity of the chromatographed amino acid spots was measured with respect to the spot intensity of the internal standard, by automated scanning fluorometry[5]. A subsequent computerized evaluation procedure, identifying the single spots as corresponding amino acids, used pre-established calibration curves to calculate the actual amino acid concentration from the measured relative spot fluorescence[5,6]. The standard deviation of this method of amino acid analysis depends on the amino acid and varies between 3 and 10%.

Determination of the amino acid activity

To measure the amino acid radioactivity, autoradiographs were prepared from the chromatograms used for the amino acid analysis. The blackening of the X-ray film was calibrated by use of labelled external standards, and the activity of the chromatographed amino acid spots was calculated from the absorbances of the corresponding autoradiographic spots.

TABLE I

ACTIVITY OF EXTERNAL STANDARDS USED FOR CALIBRATION OF THE X-RAY FILM BLACKENING

Final spot activity (determined by LSC) of the ^{14}C-labelled external standards and radioactivity of the standard solutions employed for their preparation. A 0.5-μl volume of each solution was used for preparation of the standards as described in Materials and methods.

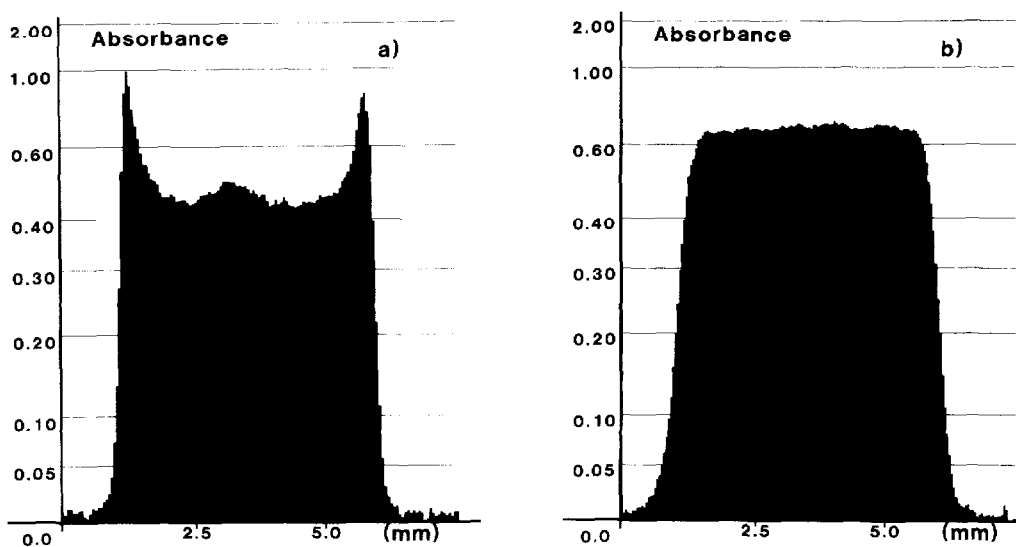| Standard	Standard activity (dpm)	Activity of the standard solution
1	40 681 $\pm$ 437	1.35 MBq/ml
2	30 984 $\pm$ 208	1.03 MBq/ml
3	21 830 $\pm$ 404	0.73 MBq/ml
4	12 348 $\pm$ 107	0.41 MBq/ml
5	6162 $\pm$ 89	0.21 MBq/ml
6	1934 $\pm$ 19	62.9 kBq/ml
7	1102 $\pm$ 13	37.0 kBq/ml
8	683 $\pm$ 26	22.2 kBq/ml
9	356 $\pm$ 8	11.1 kBq/ml
10	193 $\pm$ 4	7.4 kBq/ml
11	148 $\pm$ 9	4.8 kBq/ml
12	65 $\pm$ 4	2.2 kBq/ml

Fig. 2. Computer plot of a one-dimensional section through the absorbance of autoradiographs obtained from labelled external standards: (a) external standard without homogeneous activity distribution, due to the chromatographic effects during application of the activity to the carrier sheet; (b) external standard with an almost homogeneous activity distribution. The preparation of the external standards is described in Materials and methods.

Preparation of the labelled external standards

The blackening of an X-ray film depends on the film sensitivity, the properties of the carrier system used (gel, paper, polyamide), the number of desintegrations per s (Bq), the kind and energy of the emitted radiation and the exposure time. Therefore a careful calibration of the X-ray film blackening is essential for quantitative evaluation of autoradiographs. The calibration with respect to the activity and exposure time was done by use of external standards of defined activity. Twelve radioactive standard solutions were prepared, consisting of different amounts of L-[U-^{14}C]-phenylalanine (specific activity 1.85 MBq/mol) in bovine serum albumin (100 mg BSA per ml water). The corresponding radioactivity is given in Table I. Using calibrated micro capillaries (Drummond, Broomall, PA, U.S.A.), 0.5 μl of each solution were applied to 3 cm × 4 cm plates of the same polyamide sheet as used for the amino acid analysis described above. The "chromatographic effect" that normally occurs during application of a solution causes an accumulation of the applied substance at the border of the emerging spot (see Fig. 2a). To avoid this the polyamide sheet was moistened with water before application. For the same reason the applied standard solutions were prepared from an highly viscous albumin solution. Thus the "chromatographic effect" was minimized and an almost homogeneous activity distribution within the single standards was obtained (Fig. 2b).

In this manner 3 cm × 4 cm standard plates were produced, each one containing twelve circular (about 6 mm in diameter) standards of defined, graded activity. The mean activity of each of the twelve standards was determined. The areas of ten spots of each standard were excised, transferred to counting vials containing 1 ml water and 9 ml of scintillation fluid (Lumagel SB; LKB, Gräfelfing, F.R.G.) and
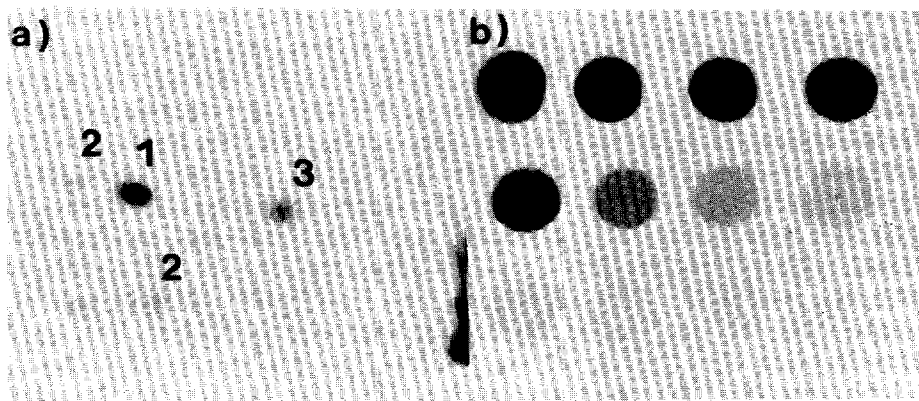
Fig. 3. Autoradiographs obtained from the ^{14}C-labelled dansylated amino acids Phe (1), Tyr (2) and Ala (3) on a micro chromatogram after TLC (a) and from twelve ^{14}C-labelled external standards (b). For preparation of the standards (standard activities in Table I) and of the chromatograms see Materials and methods.

counted in an LKB 1217 RACKBETA liquid scintillation counter. A counting efficiency of about 87% was determined by the channels ratio method. The mean activities are given in Table I. For calibration of the X-ray film blackening, these plates together with the analytical chromatograms were subjected to autoradiography.

Autoradiography

The bottom of a 18 cm × 24 cm X-ray film cartridge (Siemens, Göttingen, F.R.G.) was covered with one layer of the polyamide sheet described above. Within this sheet 24 recesses (3 cm × 4 cm) were left. The chromatogram and standard plates were placed into these recesses, thereby being fixed inside the cartridge, and the 18 cm × 24 cm X-ray film (X-OMat-S; Kodak, Stuttgart, F.R.G.) was pressed uniformly against all plates. During exposure (20, 70 or 170 h) the cartridge was kept at −70°C. After exposure the X-ray film was developed for 7 min in Kodak LX 24 X-ray developer (20°C). The development was halted with 0.01% acetic acid (1 min), and the film was fixed for 5 min in Kodak AL 4 X-ray fixer (20°C). To minimize background blackening, the X-ray film was handled in complete darkness. Fig. 3 shows autoradiographs obtained from the external standards and a thin-layer chromatogram of ^{14}C-labelled amino acids.

Quantitative evaluation of the autoradiographs

The presence of labelled amino acids on chromatograms is revealed by their autoradiographic image (Fig. 3a). In order to determine the radioactivity of the amino acid spots from the corresponding absorbance of the autoradiographic spots, the autoradiographs were scanned at 100 samples/mm^2 using a recently developed high-performance two-dimensional scanning photometer system[8], controlled by a MNC/DECLAB-23 laboratory computer (Digital Equipment). Since the relationship between the activity and autoradiographic absorbance is not linear[9], the subsequent computerized evaluation has to convert each local absorbance into a local radioac-
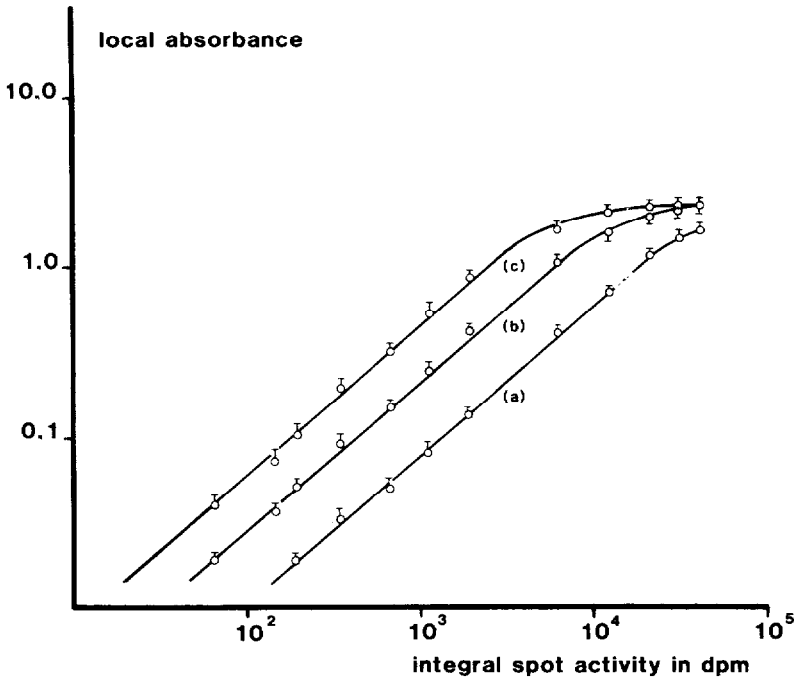
Fig. 4. Integral standard activity (determined by LSC) of the ^{14}C-labelled standards used for calibration of the X-ray film blackening and local absorbances of the resulting autoradiographic spots after (a) 20, (b) 70 and (c) 170 h of exposure. The values are mean $\pm$ S.E.M. ($n = 5$). The preparation of the standards and calculation of the local absorbances is described in Materials and methods.

tivity value. This conversion is based on the calibration of the X-ray film blackening by use of external standards.

Sections of 10 mm $\times$ 10 mm encompassing each standard were scanned (grid resolution 0.1 mm as described above). Owing to the homogeneous activity distribution within the standards, the absorbance profile corresponds to a truncated cone with a very steep slope and an almost uniform plateau (Fig. 2b). Therefore the local absorbance of the standards may be defined as the integral standard absorbance "volume" above the background divided by the standard area. It was convenient to take the number of samples with absorbance above a threshold of 0.4 times the plateau level as the standard area (plateau level = difference between average plateau absorbance and average background absorbance). Fig. 4 shows for three exposure times the mean local absorbance of the twelve standards as a function of the corresponding integral standard activity. As expected, the local absorbance increases with activity and exposure time until blackening begins to saturate at an absorbance of about 1.3.

To enable the activity calculation at each point of measurement, the local standard absorbance was calibrated as a function of the local standard activity defined as the given integral standard activity (Table I) divided by the corresponding standard area. This calibration has to be done for each exposure time applied. A calibration graph for an exposure time of 70 h is shown in Fig. 5. Small deviations
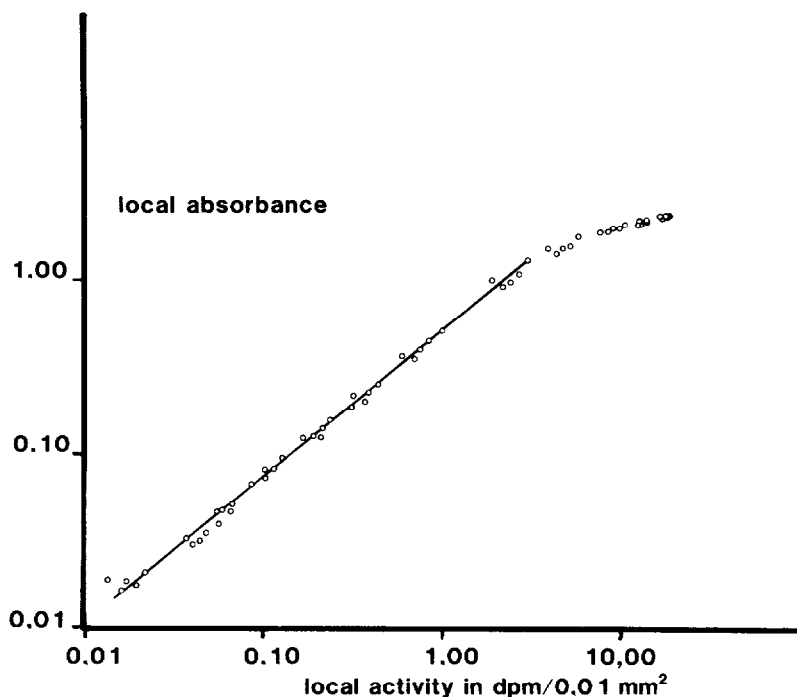
Fig. 5. Calibration graph of the X-ray film blackening for 70 h of exposure. Local absorbances of the autoradiograph are plotted as a function of the calculated local activities of the corresponding standards. Individual values are plotted to show the excellent correlation between the local activities of individual standards and the corresponding local absorbances. The preparation of the standards, the calculation of their local activities and the evaluation of the autoradiographs is described in Materials and methods.

in the size and activity of the standards used are the reason for the differences in the corresponding local activities within each group of standards. Therefore individual values are dotted in the figure to illustrate the excellent correlation between the local activity and the corresponding local absorbance. The correlation is well described by exponential regression (correlation coefficients greater than 0.997) up to an absorbance of about 1.3. Above this value the absorbance approaches a plateau due to saturation of the X-ray film blackening. If this limit is exceeded a special warning is issued by the evaluation program. In this case it is necessary to shorten the exposure time. Thus the evaluation range can be adapted to all requirements by varying the exposure time. The calibration error is about ± 3%.

The conversion of single absorbances to corresponding local activities is followed by a segmentation into background and chromatographic spots according to Zimmer et al.[5]. After background correction, the integral spot activities (dpm per chromatographic spot) are printed together with a spot map of the respective autoradiograph.

Calculation of the radioactivity of amino acids

The method described above enables the activity measurement of dansyl amino acid spots. Even when constant amino acid radioactivities are analysed, the activities
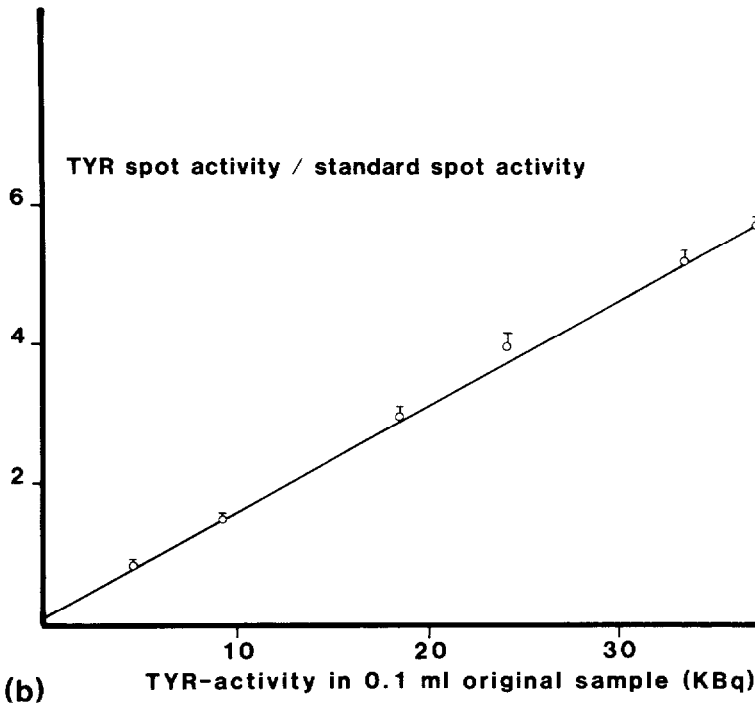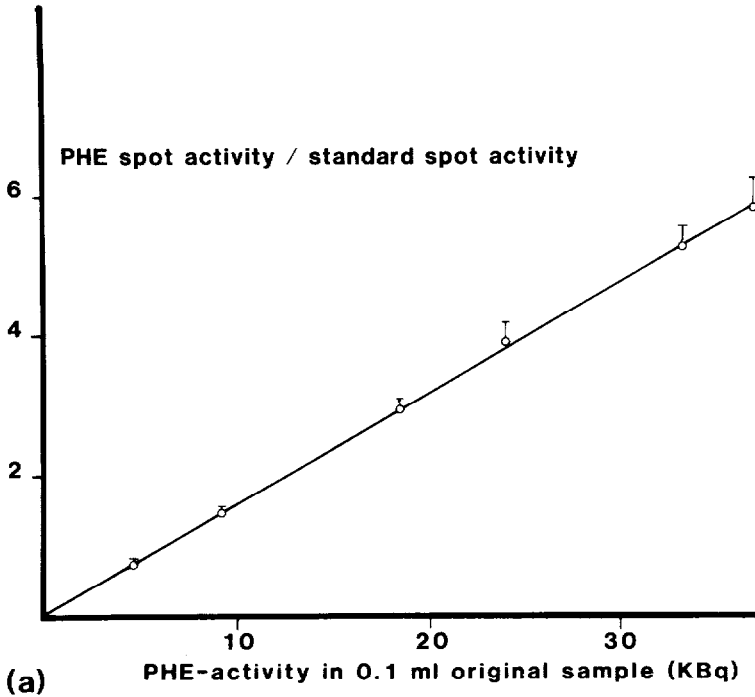
(a)

(b)

Fig. 6. Calibration graphs for calculation of the radioactivity of amino acids in the sample used to correct for the yield of the dansylation and for deviations of the chromatographic procedure. L-[U-^{14}C]-Phenylalanine (a) and L-[U-^{14}C]tyrosine (b) were dansylated and separated by TLC as described in the text. The spot activity was determined by quantitative evaluation of prepared autoradiographs. Plotted are the activities of the chromatographic spots with respect to the activity of the internal standard spot against the original activity of the respective amino acid in the sample. The values are mean ± S.E.M. Regression equations: (a) $y = 5.841x + 0.051$ ($r = 0.994$); (b) $y = 5.556x + 0.162$ ($r = 0.999$).

of the chromatographic spots vary as a function of the yield of the dansylation and the amount of dansylated sample applied to the chromatogram. Thus a calibration is required to calculate the amino acid sample activity from the corresponding chromatographic spot activity. This calibration was carried out for ^{14}C-labelled phenylalanine and tyrosine: Six mixtures were prepared, each containing unlabelled amino acids, a Ringer's salt solution and 2.5, 5, 10, 13, 18 or 20 μl respectively of the labelled amino acids L-[U-^{14}C]phenylalanine (18.648 TBq/mol, 1.85 MBq/ml) and L-[U-^{14}C]-tyrosine (18.315 TBq/mol, 1.85 MBq/ml). The final concentrations of the compounds corresponded approximately to those found in rat serum. Using D-α-phenyl[1-^{14}C]glycine as an internal standard, these mixtures were dansylated and separated by two-dimensional TLC as described above. The activities of the individual chromatographic spots were measured with respect to the spot activity of the internal standard by quantitative evaluation of prepared autoradiographs. Plots of the relative spot activities *versus* the activities of the applied amino acids are shown in Fig. 6. The correlation coefficients are greater than 0.997.

Using these calibration graphs it is possible to calculate the sample amino acid radioactivities from the spot activities of a prepared chromatogram. In principle, this method allows the simultaneous measurement of the activity of the 22 amino acids and related compounds that can be dansylated and separated by TLC. The error of measurement by this method is 2–10%.

RESULTS AND DISCUSSION

Micro TLC of dansyl derivatives has found widespread application in the separation of amino acids, peptides and glycopeptides. The procedure has several advantages. First it is very easy to handle and requires no extensive pre-purification. Secondly it enables the simultaneous separation of all the 22 amino acids on one chromatogram. Thirdly only a small amount of the sample is required, since the separation is carried out in the micro range. Finally, the short separation time of the

TABLE II

COMPARISON OF ACTIVITY MEASUREMENTS BY LIQUID SCINTILLATION COUNTING AND BY QUANTITATIVE EVALUATION OF AUTORADIOGRAPHS

Spot activities of dansylated ^{14}C-labelled amino acids on micro thin-layer chromatograms were analyzed by liquid scintillation counting of the excised spot areas and by quantitative evaluation of prepared autoradiographs. The results of individual measurements and the percentage difference of the autoradiographic method with respect to LSC are given. The preparation of the chromatograms and the quantitative autoradiography is described in the text.

Activities (dpm)		Difference (%)	Activities (dpm)		Difference (%)
LSC	Autoradiography		LSC	Autoradiography	
2393	2314	−3	596	571	−4
2524	2485	−2	615	580	−6
2339	2172	−7	513	513	0
1133	1062	−6	238	213	−10
1365	1231	−10	268	258	−4
888	872	−2	261	256	−2

micro chromatograms and the possibility of a simultaneous development of five chromatograms[10] is very time saving: the separation of five samples requires only 30 min. Based on this separation, a quantitative analysis can be achieved by computerized fluorescence evaluation of the chromatograms. The sensitivity and reproducibility of this method are similar to those of other modern separation techniques like high-performance liquid chromatography (HPLC) and the amino acid analyzer. However, a very short time is required for the analysis due to the simultaneous separation and evaluation of at least four samples[6]. Thus, more than 40 samples can be analyzed per day.

Because of these advantages it is desirable to exploit this method for the analysis of the specific radioactivity of amino acids and related compounds. As long as the amino acid radioactivity is measured by conventional LSC of excised spots, however, several difficulties arise: beside the high expenditure of time and the loss of material described above, the radioactivity of the chromatographic spots must be high enough to be detected by LSC. Therefore, in particular the analysis of very small amounts of a substance with low specific radioactivity is limited. The quantitative evaluation of prepared autoradiographs avoids these disadvantages: an autoradiograph can be prepared very easily and the evaluable range can be adapted to all requirements by variation of the exposure time. In addition, an autoradiograph can be filed easily without destroying the original chromatogram, which is preserved for other purposes.

Different methods have been reported for quantification of autoradiographs[11-14]. The method described here is very time saving because of the high scanning speed and computerized evaluation. The two-dimensional evaluation of a 3 cm × 4 cm micro autoradiograph takes only 12 min. The method is also very sensitive. Spots containing less than 0.1 Bq/mm^2 can be evaluated after an exposure time of 70 h. Finally, it is very accurate. Since the relationship between the activity and film blackening is non-linear, the activity is calculated for each absorbance at a time. Really quantitative autoradiography is possible only through this prerequisite, which according to our knowledge is considered here for the first time. Thus, the results obtained correspond very well to the real activity of the chromatographic spots; a comparison of the results obtained by LSC of the excised spots and by quantitative evaluation of prepared autoradiographs is given in Table II. By quantitative evaluation of the autoradiographs about 95% of the activity measured by LSC was detected. The mean loss of 5% is due to the procedure of data segmentation into spots and background: the background noise of the X-ray film causes a loss of detected activity at the border of the spots during segmentation. However, as long as there is a constant ratio between the spot activity and size, this can be compensated by a correction factor, which should be determined separately for each application.

These results indicate that this quantitative evaluation of autoradiographs can be used for measurement of the activity of ^{14}C-labelled spots on micro thin-layer chromatograms. It can also be applied to autoradiographs obtained with other isotopes with high emission energy or from paper chromatograms. Based on this principle, a quantitative evaluation was made of autoradiographs obtained from ^{3}H- and ^{14}C-labelled gels[15].

As described above, an internal standard can be used to correct for the yield of the dansylation and for deviations of the chromatographic procedure. This permits

calculation of the substance concentration from the fluorescence of corresponding chromatographic spots. Likewise, a labelled internal standard and pre-established calibration graphs enable the calculation of amino acid activities from the radioactivity of corresponding chromatographic spots, as shown for ^{14}C-labelled phenylalanine and tyrosine.

By combination of the results from both procedures, specific radioactivities can be calculated. In principle, this method enables a simultaneous determination of the specific activity of 22 amino acids. Normally nmol-amounts of the respective amino acids are used for analysis[7]. If necessary, these amounts can be reduced further. The activity measurement by an autoradiograph allows the determination of extremely low specific radioactivities by use of prolonged exposure times.

REFERENCES

1 V. Neuhoff, in V. Neuhoff (Editor), *Micromethods in Molecular Biology*, Springer, Berlin, Heidelberg, New York, 1st ed., 1973, Ch. 2, p. 85.
2 H. Laatsch, *J. Chromatogr.*, 173 (1979) 398.
3 D. Biou, N. Queyrel, M. N. Visseaux, I. Collignon and M. Pays, *J. Chromatogr.*, 226 (1981) 477.
4 M. Weise and D. E. Oken, *J. Chromatogr.*, 152 (1978) 175.
5 H.-G. Zimmer, H. Kronberg and V. Neuhoff, in T. Sakai (Editor), *Proceedings of the 4th Int. Joint Conference on Pattern Recognition, Kyoto, 1978*, p. 834.
6 A. M. Marx, W. Ehrhardt and V. Neuhoff, in preparation.
7 K. Schott, G. Huether and V. Neuhoff, *Biochem. Med.*, 29 (1983) 285.
8 H. Kronberg, H.-G. Zimmer and V. Neuhoff, *Clin. Chem.*, 30 (12) (1984) 2059.
9 R. A. Laskey and A. D. Mills, *Eur. J. Biochem.*, 56 (1975) 335.
10 P. Seiler, W. Thorn and V. Neuhoff, *Funkt. Biol. Med.*, 3 (1984) 85.
11 S. A. M. Cross, A. D. Groves and T. Hesselbo, *Int. J. Appl. Radiat. Isot.*, 25 (1974) 381.
12 M. Suissa, *Anal. Biochem.*, 133 (1983) 511.
13 J. C. Garrison and M. L. Johnson, *J. Biol. Chem.*, 257 (1982) 13144.
14 N. L. Anderson, J. Taylor, A. E. Scandora, B. P. Coulter and N. G. Anderson, *Clin. Chem.*, 27 (1981) 1807.
15 V. Neuhoff and R. Stamm, in M. J. Dunn (Editor), *Electrophoresis '86*, Verlag Chemie, Weinheim, 1986, p. 376.