Computed Resolution and Relative Specific Radioactivities of Radiolabelled Proteins Synthesized by Isolated Gastric Mucosal Cells

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1. By radiolabelling, polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and fluorography, more than 50 soluble proteins in the molecular-weight range 15 000–100 000 were shown to continue to be synthesized after cells had been isolated from rat gastric mucosa. 2. Densitometric measurements of stained gels and fluorographic films were processed by computer to resolve individual overlapping Gaussian peaks corresponding to the protein bands. 3. Comparison of resolved peak areas of radioactivity and staining showed certain bands to have characteristically high relative specific radioactivities. 4. The computer programs (in FORTRAN) permit the analysis of a single densitometric trace or the simultaneous comparison of a corresponding pair of densitometric records of stained gels, or of fluorographic films, or a combination. Central processing unit time is used economically. 5. The programs identify the Gaussian components that contribute to the records and estimate their means, standard deviations and enclosed areas. These estimates are improved by a piecewise iterative method that minimizes the errors between the calculated and the experimental data. 6. Relative specific radioactivities are calculated as the normalized ratio of the area of a fluorographic film peak and the area of the corresponding stained gel peak. The computer programs have been deposited as Supplementary Publication SUP 50094 (55 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms given in *Biochem. J.* (1978) 169, 5.

Isolated cells of gastric and intestinal mucosa provide a useful model system in which to study short-term biosynthesis, metabolism and responses to drugs or environmental stimuli. Gastrointestinal mucosal cells *in vivo* are liable to experience transient high concentrations of drugs and toxic substances. Isolated cell preparations contain a representative population of specialized and differentiated cells. An important question, when cells have been isolated by the use of Pronase or other potentially traumatic procedures, is whether they are viable and retain a reasonable spectrum of biochemical functions. Viability is sometimes expressed in terms of the ability of cells to exclude Trypan Blue or other dyes, their respiration rate, retention of cytosol enzymes or other criteria (see, e.g., Thieden *et al.*, 1976). We have investigated the biosynthesis of proteins in gastric and intestinal cells, partly as a much more comprehensive indicator of the health of the cells than quantal tests provide, and partly through our interest in the effects of local concentrations of drugs and other compounds on the synthesis of total and specific proteins in the gastrointestinal mucosa.

The overall technique involves the preparation of

suspensions of isolated cells, incubation with ^{14}C -labelled amino acid, then separation of proteins by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate. Proteins are detected by staining the gels, and their radioactivity is detected by fluorography. The intensity of staining and the amount of radioactivity in each protein band are measured by densitometry of the gel and of the fluorographic film. This paper describes a rapid procedure for resolving the densitometric measurements into constituent peaks in order to make quantitative comparisons between experiments and to enable the relative specific radioactivity of single bands to be determined.

The computer program automatically identifies the Gaussian components present in a densitometric record of the fluorographic film or stained gel, and estimates the means, standard deviations and enclosed areas of these components. Relative specific radioactivity of any band can then be calculated as the ratio of the normalized areas of radioactivity and staining. An interactive program is also provided that compares two complete densitometric records and measures ratios automatically.

Experimental

Isolation of gastric mucosal cells

Adult Wistar rats weighing about 250 g were killed by concussion and exsanguination. The stomach was removed, everted as described by Dikstein & Sulman (1965) and filled with Pronase (Merck, D-61 Darmstadt, Germany) at a concentration of 1000 PUK units/ml in contact with the serosal surface, as described by Lewin *et al.* (1974). The remainder of the procedure was as described by Lewin *et al.* (1974), except as follows. The medium in which the stomach was incubated for three periods of 30 min at 37°C was minimum essential medium for suspension cultures (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) plus 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 2 mM-EDTA, 100 µg of gentamicin (Flow Laboratories)/ml and 30 mg of Dextran T40 (Pharmacia G.B. Ltd., London W.5, U.K.)/ml, pH 7.35. The medium in which cells were detached from the stomach by stirring was the same as above with 5 mM-NaHCO₃, 1 mM-CaCl₂ and 30 mg of albumin/ml, but without Dextran or EDTA. All adjustments of pH were made with HCl or NaOH.

Aseptic precautions

Apparatus was sterilized by autoclaving at 100 kPa above ambient pressure (at 121°C) or, for plastics, with 70% (v/v) ethanol. Media and solutions were aseptically filtered through membranes of pore size 0.22 µm. Gentamicin was present in all media at 100 µg/ml.

Protein biosynthesis

Approx. 5×10^7 cells were washed four times by centrifuging at 1000g for 1 min and resuspending in incubation medium to the original volume (9 ml), or to 2.0 ml finally. Incubation medium was leucine-free modified Eagle's medium with Earle's salts (Gibco Bio-Cult, Paisley, Scotland, U.K.) (80%, v/v), with Hepes (15 mM), gentamicin (100 µg/ml) and albumin (30 mg/ml). To this was added 10 µCi of L-[U-¹⁴C]-leucine, final specific radioactivity 200 mCi/mmol (The Radiochemical Centre, Amersham, Bucks., U.K.). The 25 ml Erlenmeyer incubation flasks were flushed with O₂/CO₂ (19:1) every 30 min and shaken at 2.5 cycles/s with 2 cm amplitude for 3 h at 37°C.

Cells were recovered by centrifugation at 1000g for 1 min, then washed to remove most albumin by resuspending and centrifuging from 10 ml of incubation medium with albumin omitted. The pellet was frozen and thawed four times at -196°C and 37°C, then thoroughly disrupted for 2 min at 0°C with 10 mM-sodium phosphate buffer, pH 7.4, in an MSE (Crawley, Sussex, U.K.) homogenizer with a Jencon Scientific (Hemel Hempstead, Herts., U.K.) glass container. The homogenate was centrifuged at

9300g for 5 min and the supernatant separated from the residue.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Slab 12.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate were prepared as described by Laemmli (1970) and electrophoresed in an apparatus as described by Studier (1973). Samples were treated with 2% (w/v) sodium dodecyl sulphate and 5% (v/v) mercaptoethanol as described by Laemmli (1970). The sample loaded on the gel illustrated in Fig. 1 contained supernatant extract equivalent to 1.2×10^6 cells with a dry weight of 0.3 mg, and contained 1.5×10^4 d.p.m. of radioactivity. A constant current of 30 mA was applied to a gel 1.5 mm thick and 100 mm wide, migration being from cathode to anode. Proteins or subunits were stained by gently agitating the gel for 90 min in methanol/acetic acid/water (5:2:23, by vol.), containing 0.1% Coomassie Blue R250 (Raymond Lamb, London N.W.10, U.K.), followed by destaining of the background in 10% (v/v) acetic acid. Standards, and their accepted subunit molecular weights, were: rabbit muscle phosphorylase *a* (94 000); bovine serum albumin (68 000); horse liver alcohol dehydrogenase (43 000); pig heart lactate dehydrogenase (36 000); bovine pancreas chymotrypsinogen A (25 635); horse heart myoglobin (16 890); egg lysozyme (14 300); aprotinin (6500). Cytochrome *c* (12 000) migrated with lysozyme. Bands will be referred to this scale for nominal purposes only, since electrophoretic mobility may depend on molecular character as well as size.

Radioactivity in gels

Radiolabelled protein bands were detected by the fluorographic film-detection method of Laskey & Mills (1975), or by direct radioautography.

Densitometry

A Kipp and Zonen (Delft, Netherlands) micro-densitometer was used to measure the intensity of stain in wet gels and the profile of radioactivity registered by the fluorographic film.

Computer analysis of densitometric data

The programs used have been deposited with the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, as Supplementary Publication SUP 50094. A description of their design and use, running to 2000 words, is included in the Supplementary Publication. A brief summary is provided below.

The program is arranged to be economic in central processor time. It avoids the necessity, inherent in some alternative procedures, for a preliminary subjective assessment of the densitometric records to

provide an initial estimate of the three parameters that describe each Gaussian distribution present (Booth & Kenny, 1976).

The densitometer traces are digitized at positions equispaced in the x -direction, and the densitometric trace of radioactivity is corrected for non-linearity. A minimum of six data points must be input for each peak in the densitometric trace. The curve is analysed by a computer-program adaptation of the graphical method of Bhattacharya (1967). This determines the position and width of peaks in the densitometer trace and estimates the areas contained by the Gaussian distributions. A piecewise iterative method is used to make slight adjustments to these estimates of mean, standard deviation and enclosed area of the peaks to minimize the deviations between the calculated and the input data functions. The output is a listing of the mean, standard deviation and enclosed area of each peak. The area is expressed in absolute units and also as a percentage of the total area enclosed by the trace. This is followed by a summary of the total error and total enclosed area and a listing of the data input, the best-fit function and the error function. An ancillary program will read this output listing and present the data in graphical form. This is not included in the Supplementary Publication, since it would generally need to be rewritten to suit local equipment.

An extended version of the program allows a pair of equivalent densitometric records to be analysed and the resolved peaks of one expressed proportionately to the corresponding peaks of the other. The program is interactive between the traces in order to optimize the registration of corresponding peaks. Data index positions for two corresponding and readily identifiable marker peaks that are present in both traces are input. If necessary, the program initially lengthens the shorter trace and linearly interpolates data points and adjusts their positions for an exact point by point registration between the traces. The traces are normalized to equal total areas and added point by point. This composite distribution is analysed as above by the method of Bhattacharya (1967). This determines the position and width of peaks that may occur in either one or both of the traces. The two traces are then treated separately and estimates are made of the characteristics of the Gaussian distributions as described above.

Results and Discussion

Summary of favourable conditions for protein biosynthesis

The gastrointestinal tract of ordinary laboratory animals is heavily colonized with bacteria that grow extremely rapidly at 37°C in aerobic tissue-culture media. Neither penicillin with streptomycin, nor kanamycin, chloramphenicol or rifampicin at con-

ventional concentrations effectively inhibited all bacterial activity. Gentamicin did, however, and was adopted for routine use, since its specificity is very broad (Rudin *et al.*, 1970) and it is unaffected by extremes of pH (Schafer *et al.*, 1972). Various types of cultured cells have been shown to tolerate gentamicin at concentrations 10–60-fold higher than the 100 µg/ml used here, cytotoxic concentrations being higher still and typically 3–6 mg/ml (Schafer *et al.*, 1972). In our tests with a mixed bacterial culture derived from rat stomach, and with *Escherichia coli*, growth was suppressed with as little as 1 µg of gentamicin/ml. At 1–1000 µg/ml, gentamicin had no effect on total protein biosynthesis in isolated gastric cells.

The freshly prepared cells needed to be centrifuged and resuspended three or four times to remove Pronase, otherwise they synthesized protein for 2–3 h but then underwent autolysis and proteolysis (Trotman, 1977). A concentration of 3% (w/v) albumin in the medium enhanced protein biosynthesis compared with 0–1% (w/v) albumin, and under optimum conditions synthesis continued for 5–8 h after removal of the stomach (Trotman, 1977).

Separation of radiolabelled proteins

Incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable material is insufficient evidence that the cells, after exposure to Pronase and EDTA, retain a regulated system for the biosynthesis of proteins. If the mechanisms by which protein biosynthesis is controlled and regulated were disturbed, the cells might assemble an amorphous range of polypeptides of random length. The molecular-weight distribution of the newly synthesized polypeptides was therefore examined.

By staining of sodium dodecyl sulphate/polyacrylamide electrophoresis gels, typically between 50 and 60 proteins or subunits were detected. These stainable proteins in the post-9300g supernatant are representative of the cells before radiolabelling, since the pattern was the same if biosynthesis had been inhibited with 640 µM-cycloheximide.

Radioautographs or fluorographs of dried stained gels revealed a pattern of radiolabelled bands closely similar in position to the stained bands. Fluorography is more sensitive than radioautography, but the latter technique, since it did not obscure the pattern of staining on the dried gel, allowed the developed film record of radioactivity to be re-aligned precisely on the stained gel. Radioautographs were used therefore to resolve any ambiguities in the matching of fluorographic and stained bands. Virtually all radiolabelled bands could be assigned to corresponding stained bands, and vice versa, although the possibility remains that a radiolabelled and stained pair of bands coinciding in electrophoretic mobility are not identical molecules. It appears, however, that these cells,

despite their rigorous treatment *in vitro*, continued to synthesize a full complement of specific proteins within the molecular-weight range examined. By this criterion the cells retained a high degree of viability and biochemical function.

Relative specific radioactivities of protein bands

Although labelled and stained bands coincided in apparent molecular size, they differed in relative prominence. From visual comparison of stained gels and fluorographs, some bands, such as those of 91 000-, 43 000-, 25 000- and 19 000-mol.wt. protein, were invariably more prominent in their radio-labelling than in stained appearance (Fig. 1). The characteristic distribution of [^{14}C]leucine among the different bands was highly reproducible in successive preparations of cells, providing further evidence for a regulated process of protein biosynthesis.

Relative specific radioactivities can be estimated by comparing simultaneous values of the two densitometric records of radioactivity and staining (Table 1).

The validity of the parameter depends on the assumption that the response of the photographic emulsion is proportional to radioactivity, which is achievable by fluorography on sensitized film but not by radioautography (Laskey & Mills, 1975). A further assumption is that stain intensity is related to the amount of protein in the band, which is valid within limits (Weber *et al.*, 1972), but may be less so in gels thicker than 1.5 mm or if staining is prolonged. Different proteins may have different affinities for stain, but since the majority of bands tend to be unknowns the extent of this problem is unpredictable.

Protein labelling depends on leucine content, which is also a little unpredictable when one is dealing with unknowns, although in our system the similarity of results with [^{14}C]proline and [^{14}C]leucine labels showed none of the labelled proteins to be markedly anomalous in their content of either amino acid. In any case, experimental differences observed in the labelling of a given band will have a consistent basis of comparison. Experimental inconsistencies affect-

Table 1. *Relative specific radioactivities of some proteins of isolated gastric mucosal cells labelled with [^{14}C]leucine*
Bands detected by staining and fluorography on sodium dodecyl sulphate/polyacrylamide electrophoresis gels are indexed nominally by reference to a scale of marker proteins as described in the text. Manually calculated values of relative specific radioactivity were calculated from simultaneous readings of radioactivity and stain from densitometric records. Computed values were calculated from the areas of the two peaks after resolution into separate Gaussian distributions. In each case the values are normalized to the integrated area of the complete trace.

Nominal mol.wt.	Protein band		Relative specific radioactivity	
	Radioactive-band serial number	Stained-band serial number	From simultaneous densities (manual)	From resolved areas (computed)
91 000	7	11	1.80	4.77
84 000	9	12	0.70	0.24
79 000	10	13	1.08	1.32
47 000	22	27	1.00	0.66
43 000	24	28	2.44	1.29
25 000	33-37	40	3.61	3.28
19 000	44	50	1.82	17.1
17 000	46	54	0.62	0.67
15 000	50	59	1.15	0.98
12 000	52	63	0.54	0.98

Fig. 1. *Supernatant proteins of isolated gastric mucosal cells labelled with [^{14}C]leucine*

(a) Photograph of a fluorographic image of radiolabelled proteins separated by electrophoresis on sodium dodecyl sulphate/polyacrylamide gel. (b) includes a photograph of the same gel stained with Coomassie Blue R250. The fluorographic film and the wet stained gel were each scanned in a densitometer, then the records were digitized and processed by computer. The diagram accompanying each photograph is the graphical summary of the computer output listing. The broken-line trace represents the input trace as reconstructed from the digitized input. The continuous trace is the summation of the resolved Gaussian peaks, which are shown below. For clarity, input and summation traces are displaced upwards by one and two units respectively and resolved peaks are restricted to $\pm 1\text{s.d.}$ The remaining differences between the input and summation traces can be eliminated by resolving smaller peaks, but a threshold value of individual peak area has been applied as discussed in the text. The vertical scale of absorbance is arbitrary. The scale below each abscissa is the resolved peak serial number. The scale of molecular weights ($\times 10^{-3}$) above the lower diagram shows the working identities assigned to certain bands for nominal purposes. Migration was from left (cathode) to right.

ing, for example, the activity of a batch of cells, efficiency of staining, efficiency of fluorography or extent of photographic development, have no effect on relative-specific-radioactivity calculations, provided that the derived values are normalized to the integrated area of the complete densitometric trace.

Subject to these limitations of interpretation, relative specific radioactivity can be calculated more accurately by first extracting the profile of a given band from the summation curve obtained by densitometry. The calculation can then be done by comparing the areas of the two peaks. This is the purpose of the program and an example of its application is shown in Fig. 1; some computed values are compared with manually calculated values in Table 1.

Since the resolving technique is superior to the data, it needs to be applied with discrimination and due recognition of the limitations of the data, especially where the number of bands is large. It is evident from occasional clear regions between bands that the baseline of density within the migration channel is always higher than that of spare areas of gel, both by staining and fluorography, and therefore subject to slight uncertainty. The method assumes fundamentally that the peaks are Gaussian, therefore any skewed distributions will be resolved into a main peak and small subsidiary peaks. Peak distortion can also originate in the time constant of the densitometer response. Similarly, broad saturated areas or regions of elevated plateau are liable to be resolved as a series of Gaussian distributions. For these reasons it is sometimes apparent that certain output peak areas must be added to reconstitute a particular input band.

Imperfections in the data also lead to the generation of numerous very small peaks. The broken and solid continuous traces shown in Fig. 1 are respectively the input data and the summation curve attained by addition of the series of unresolved peaks. These two traces match if no lower limit of acceptable peak area is imposed. Instituting such a threshold value eliminates small peaks and decreases computer processing time at the expense of a corresponding residual difference between the input curve and the resolved trace. Choice of the threshold value is a local problem depending on the type of raw data, as curves other than densitometric records of electrophoresis gels can be analysed in the same way.

The interactive program for comparing all the resolved peaks of two traces automatically is particularly applicable to problems of monitoring responses of specific bands to experimental differences, for example by comparing the protein-labelling profile of treated cells with control cells. Stained proteins can be compared similarly. The fine resolution of

polyacrylamide-gel electrophoresis imposes limits on the application of this technique, because inconsistency between migration tracks could set corresponding bands out of step. It is highly desirable for comparisons to be made on the same slab gel, and in adjacent tracks well clear of the sides. The program allows adjustment of track lengths if required. This interactive program has also been applied to express relative specific radioactivities by comparing fluorographic with stain traces, but again caution is dictated by the possibility of discrepancies arising out of shrinkage and distortion of the gel during impregnation and drying.

These programs have their chief applications where we are attempting to quantify experimental differences in relative specific radioactivity of particular proteins, reflecting a change in the rate of synthesis, turnover or secretion. Changes in bands of 43000- and 25000-mol.wt. protein have been found in isolated cells treated with therapeutic antibiotics, and the technique has been applied to the problem of characterizing the synthetic activities of different fractions of cells separated from the mixed population by gradient sedimentation.

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