of cytochromic respiration could occur during a span of 15 h of incubation.

Discussion. We have found that both rates of respiration and germ tube outgrowth were strongly enhanced in conidia at 37–41 °C compared to 25–30 °C. At 25 °C, the general pattern of oxygen uptake was the same as that measured by others[13,14,15], while at 37 °C it followed the rate of outgrowth, attaining its maximum by the end of that stage 1 h earlier than at 25 °C. At its peak of activity at 37 °C, the anticipated oxygen uptake was close to that measured at 25 °C. However, 30% of the high respiration at 37 °C was found to be rerouted toward its cyanide-insensitive or alternate oxidase pathway. It is known that the partitioned electron flow during respiration is regulated so that 'the more efficient cytochrome chain operates at maximal activity and the less efficient alternate oxidase accomodates the surplus'[5]. This suggests that in conidia treated with moderate heat (37–41 °C), the stimulated respiratory rate leads to a transient saturation of the cytochromic pathway, which is compensated by the operation of the alternate oxidase or SHAM-sensitive pathway. The optimal proportion of rerouting (30% alternate oxidase activity) was obtained at 37–41 °C, in parallel with the highest rates of germination. A higher participation (70%) of the alternate pathway in 43 °C treated conidia was also found in the 'poky' strains of *N. crassa*[16]; it still allows a fair rate of emergence but slows down the elongation rate of the hyphal tube.

When the cyanide-sensitive pathway practically disappears, as occurs after 8 h at 46 °C, no germ tube can emerge any more. It has been shown[6] that the functioning of the cyanide-insensitive pathway leads to an acidification of the hyphal cytoplasm, probably by an increased compensatory glycolysis. We suggest that such acidification, noticeable in 46 °C overswollen conidia stained with permeating pH-indicators such as bromocresol green and alizarin yellow S (Turian, unpublished observations) might be related to the slowed mitochondrial reintrusion of protons related to the less efficient ATP synthetic activity allowed by that pathway. Also, the fact that such overswollen conidia are devoid of the 'proton sink' of an emerging acid tip, might contribute to the maintenance of the generalized, non-polarized internal acidity.

How then could the temperature-modulated ratio of the two types of respiration optimize the rate of germ tube outgrowth? For now, we can only suggest that at the maximal rate of respiration reached at 37–41 °C, the transient and moderate activity of the alternate oxidase might stimulate the rate of germ tube outgrowth through its early acidification of the cytosol in the swollen conidia. This would be further enhanced by the vectorial extrusion of protons from the mitochondria fronting the site of germ tube outgrowth through stimulated ATPase[9], and further relay by the increased activity of the cytochromic pathway (proton translocation by cytochrome oxidase[17]). Such polarized acidification at the site of germ tube outgrowth[8] would be counterbalanced by the relative alkalinization of the spore body cytosol resulting from the proton reintrusion required for enhanced ATP synthesis in the fully recoupled mitochondria of the germinated conidia.

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Zone immunoelectrophoresis assay applied to α_1-acid glycoprotein secretion by isolated rat hepatocytes

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Summary. A method for measuring proteins in low concentrations applying the zone immunoelectrophoresis assay is reported. The low detection limit makes it possible to measure α_1-acid glycoprotein in rat serum and also to quantify the secretion of this protein after concentration of the incubation media containing less than 10^7 isolated rat hepatocytes. The method is simple and consumes very small quantities of antiserum.

Key words. Rat serum; rat hepatocytes; α_1-acid glycoprotein; zone immunoelectrophoresis.

An in vitro system of isolated rat hepatocytes is well suited to the study of the synthesis and secretion of proteins from liver without interfering with the catabolic process. But although it is easy to measure the rate of secretion of some major proteins such as serum albumin, it is far more difficult to determine the amount of minor proteins such as α_1-acid glycoprotein (α_1-AGP). The usual techniques, such as the electroimmunodiffusion method of Laurell[1] and the radial immunodiffusion of Mancini[2] are not sensitive enough. Methods such as radioimmunoassay allow the detection of proteins in very low concentrations, but call for the use of radioactively labelled compounds and a specific spectrometer. Recently, Vesterberg[3] has

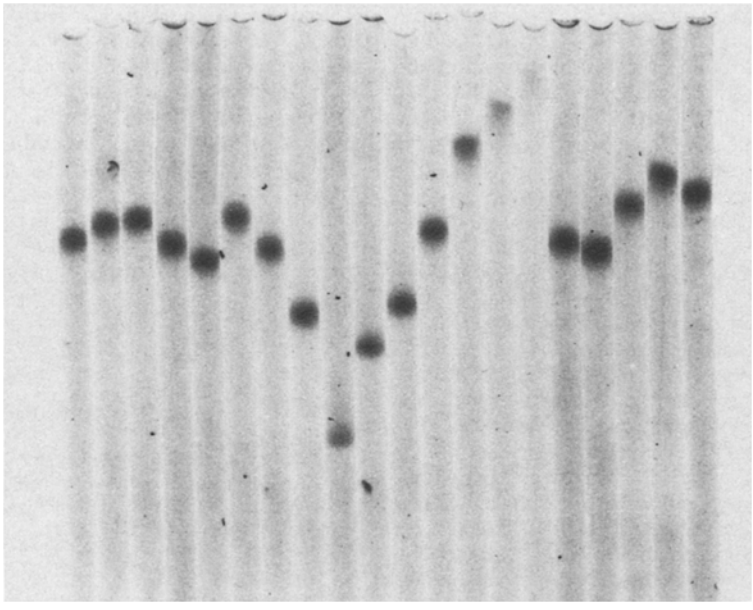

Figure 1. Typical results after ZIA and staining of the α_1-acid glycoprotein immunoprecipitate. Migration from top (cathode) to bottom (anode).

developed a simple technique, zone immunoelectrophoresis assay (Z.I.A.). Applied to human serum albumin, it allows a detection limit as low as 50 µg of protein/l. This paper deals with the conditions of quantifiying α_1-acid glycoprotein in rat serum applying zone immunoelectrophoresis, and concludes that ZIA is a reliable method which can be easily applied to measure the rate of protein secretion using no more than 10^7 rat hepatocytes. The incubation media needed for a 40-fold concentration, and the exact factor of concentration, calculated by measuring is the amount of albumin before and after concentration.

Materials and methods. Cell isolation and incubation conditions. Isolated hepatocytes were prepared from male wistar rats (350–400 g) according to the technique of Berry and Friend[4] modified by Davy et al.[5]. Cell viability was assessed by the trypan blue exclusion method and was between 80 and 90%. 5 ml of hepatocyte suspension (1.5×10^6 cells/ml) was incubated in collagen-coated plates at 37 °C in a humidified mixture of 5% CO_2/95% air, with Krebs-Ringer Bicarbonate buffer pH = 7.4. After 30 min, the medium was discarded and 5 ml of Eagle's Minimum Essential Medium (Gibco Biocult), supplemented by Non Essential Amino-acids (Gibco Biocult) and 10 mM/l of L-lactate, were added.

Cells were incubated for 3 h under the same conditions. Then the supernatant was removed for protein determinations and attached cells were resuspended in collagenase buffer for counting.

Materials and reagents. Incubation media were concentrated about 40-fold in a concentrator B 15 MINICON (AMICON). Rat α_1-AGP was isolated from the serum of adult male rats by chromatography on DEAE-Trisacryl and CM 52 cellulose columns according to the procedure of Shibata[6]. Antiserum against rat α_1-AGP was prepared in rabbits (Fauve de Bourgogne) according to the procedure of Livingston[7]. The antibody titer was 0.170 mg α_1-AGP/ml antiserum. Zone immunoelectrophoresis was performed using a Quantiphor (Desaga-Heidelberg FRG) and commercial reagents supplied by Desaga. Antiserum was used at 16 µl/ml of gel. A normal rat serum, calibrated for α_1-AGP and stored at −20 °C, was used to plot the standard curve which ranged from 0.36 mg/l to 3.6 mg/l. Final dilutions of standards and samples were made in the electrophoresis buffer supplemented by 50 µl/ml of glycerol and 1 mg/ml of bromophenol blue. 50 µl of these dilutions were pipetted on the top of each gel. Usually α_1-AGP electrophoresis were done for about 20 h with 12 V and 0.8 mA.

Results and discussion. Electrophoresis conditions. Antibody concentration was chosen to obtain the lowest detection limit with our staining procedure. An attempt was made to show by experiment the widest possible range for antigen concentrations. The highest point of the calibration curves corresponds to a zone distance of about 30 mm. Calibration curves do not change as long as the product V × h remains constant (V; voltage and h electrophoresis duration).

Nevertheless, moving towards a higher electric field, we have observed a decreasing contrast in the visualization of the immunoprecipitates. For practical reasons, electrophoresis duration was usually fixed at 20 h but in some experiments with a duration of 3 days and an unchanged voltage, we observed longer migration distances, but also a spreading of the front of the immunoprecipitate.

The characteristics of immunoprecipitates and calibration curves. Precipitates appear in the form of 1 or 2-mm-wide stained

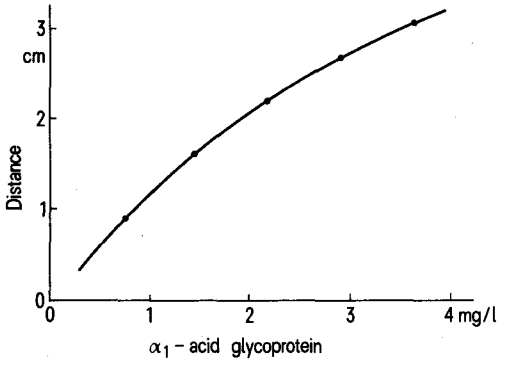

Figure 2. Typical calibration curve for quantification of α_1-acid glycoprotein.

bands. They are very dense in the middle and show symmetrical zones wich diffuse edges (fig. 1). To maximize accuracy, distances are measured from the point of application of the sample up to the most dense section. The calibration curve shown in fig. 2 becomes slightly flattened with growing concentrations. With a migration duration in excess of 46 h, the curve becomes linear but the precipitate is more diffuse.

Precision of the method. The migration distances can be measured with a margin of ±0.1 mm over the whole range of values. Using different dilutions of sample in the same run of about 10 experiments, the relative standard deviation was 2.12%, suggesting a fairly low relative error of measurements.

α_1-AGP determinations. ZIA is a simple method for calibrating proteins in high amounts, for example in rat serum. The dilutions needed for the samples are easy to find because the calibration curves spread over a large range of concentrations. The α_1-AGP concentration for normal rat serum is 182 ± 44 mg/l, (23), (mean ± SD, (n)). The measurement of the α_1-AGP concentration in the incubation medium requires a 40-fold concentration. Since the albumin concentration of the incubation media can be directly measured by ZIA, it is possible to use its determination before and after concentration in order to calculate the factor of concentration; this method gives much more reliable results than those obtained by evaluating the initial and final volumes. For 3 h the albumin and α_1-AGP syntheses are 5.16 ± 1.32 pg/hepatocyte,[5] and 0.115 ± 0.26 pg/hepatocyte,[5] respectively.

Zone immunoelectrophoresis assay allowed us to carry out the quantification of a poorly immunoreactive protein (α_1-AGP), which was not previously possible by traditional methods. The detection limit is so low that it is possible to apply this technique to give evidence of a decrease in protein secretion by hepatocytes from rats in a pathological state (diabetes, cirrhosis, etc.). Furthermore, ZIA consumes very small quantities of antiserum and the working procedure is simple and fast.

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