is the factor. This is simply because the larger the numerical value of the denominator the smaller is the value of the fraction, especially when squared.

There is no entirely satisfactory solution to this problem, other than the highly recommended procedure of never using single sets of observations, but it has been found that the mean of experimental and calculated values is a normalizing factor that considerably decreases the bias and provides a fairly satisfactory neutral weighting between large and small experimental variables. The form of each deviation term is thus:

$$4[(x_{\text{calc.}} - x_{\text{exp.}})/(x_{\text{calc.}} + x_{\text{exp.}})]^2$$

This work was carried out with the aid of a grant from the Science Research Council.

Inhibition by Salicylate of Porcine Lactate Dehydrogenase Isoenzyme 5

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Salicylate has been shown to inhibit a number of dehydrogenase enzymes by competing with the NAD molecule for its binding site at the active centre (Hines & Smith, 1964). The work now reported is part of an investigation to determine with which part of the NAD site on lactate dehydrogenase the salicylate molecule is competing. During this study evidence was obtained that the pattern of inhibition of porcine lactate dehydrogenase isoenzyme 5 by salicylate is more complex than previously (Dawkins, Gould, Sturman & Smith, 1967) reported.

Lactate dehydrogenase activity was measured during L-lactate (17–22mM) oxidation at pH 10 at 25°C by following extinction changes at 340nm. The reversibility of salicylate inhibition of lactate dehydrogenase, as reported by Dawkins *et al.* (1967), was confirmed for isoenzyme 5 by using the method of Ackermann & Potter (1949).

The inhibition by salicylate at concentrations up to 18mM with varied NAD⁺ concentrations was predominantly competitive, but some effect on the intercept of the reciprocal plot was noted, indicating that, although the major inhibitory effect arose from the combination of the inhibitor with the free form of the enzyme, some combination with the enzyme-NAD⁺ form did occur. This assumes an ordered mechanism for lactate dehydrogenase, in which NAD⁺ is the leading substrate (Novoa & Schwert, 1961; Zewe & Fromm, 1962).

The intercept replot against salicylate concentration shows considerable variance, but a definite

deviation of the gradient from zero was noted, indicating combination of salicylate with the enzyme-NAD⁺ form. This intercept effect was noted at L-lactate concentrations as high as 44mM, a result not expected were combination of inhibitor occurring at the lactate-binding site.

The slope replot against inhibitor concentration consistently produced a parabola even under conditions of constant ionic strength. The parameters of these parabolas were determined by using a weighted least-squares analysis (Cleland, 1963). This observation can be explained on the assumption that up to 2 molecules of salicylate/catalytic centre were combining with the same form of the enzyme as did NAD⁺.

The weighted mean values of K_{i1} and K_{i2} , the stepwise dissociation constants of the enzyme-inhibitor complexes EI and EI₂, were (means ± s.e.m.) 7 ± 1 and 7 ± 1mM respectively. It can be shown that the relative magnitudes of these two values may indicate that the two molecules of salicylate bind by a sequential mechanism, the binding of the first causing unmasking of the second binding site.

- Ackermann, W. W. & Potter, V. R. (1949). *Proc. Soc. exp. Biol. Med.* **72**, 1.
 Cleland, W. W. (1963). *Nature, Lond.*, **198**, 463.
 Dawkins, P. D., Gould, B. J., Sturman, J. A. & Smith, M. J. H. (1967). *J. Pharm. Pharmacol.* **19**, 355.
 Hines, W. J. W. & Smith, M. J. H. (1964). *Nature, Lond.*, **201**, 192.
 Novoa, W. B. & Schwert, G. W. (1961). *J. biol. Chem.* **236**, 2150.
 Zewe, V. & Fromm, H. J. (1962). *J. biol. Chem.* **237**, 1668.

Liver Perfusion with a Closed Gas Circuit

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An apparatus for the perfusion of isolated rat liver has been constructed in which the gas and the perfusate are both recycled in closed circuits. Temperature control is achieved by circulating water from a thermostatically controlled bath through the water jackets of the component parts of the apparatus. The medium, 50ml of Krebs-Ringer bicarbonate buffer containing 4% (w/v) of bovine serum albumin, is equilibrated with the O₂+CO₂ gas phase by passing it over a series of rotating discs on which the medium spreads in a thin film. This system gives efficient gas exchange with negligible frothing. The apparatus is fitted with oxygen electrode, flow meters and a gas-flow radioactivity counter.

At commencement of an experiment the gas reservoir of 2.5 litres capacity is filled with $O_2 + CO_2$ (95:5). This provides adequate O_2 for liver tissue for several hours, since O_2 consumption is reported to be $2.2 \mu\text{mol/min per g}$ (Schimassek, 1963). On this assumption 25 ml of CO_2 will be produced in 1 h in an 8 g liver. Therefore in a closed-circuit system at the end of 4 h the gas composition will be $O_2 + CO_2$ (91:9). This increase in CO_2 will affect the bicarbonate-buffered medium, but the minor change in pH is still within the physiological range.

The apparatus described has been found to maintain a liver in a metabolically active condition as shown by gross appearance, bile flow rate, perfusate flow rate, O_2 consumption, perfusate K^+ concentration, bromsulphthalein clearance rate, liver water content and the linear utilization and production of metabolites.

This work was supported by a grant from the Medical Research Council.

Schimassek, H. (1963). *Biochem. Z.* **336**, 460.

Evidence for an Atypical Binding of Haem in a Protozoan Cytochrome

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A haemoprotein isolated and purified from *Crithidia oncopelti* was found to have a pyridine haemochrome peak at 553 nm (2.1 M-pyridine-0.08 M-sodium hydroxide). This lies between those of protohaem-containing proteins and cytochromes c.

The haem could not be removed by acid-acetone treatment, but was readily removed by mercuric chloride in acid (Sletten, Dus, Klerk & Kamen, 1968). The pyridine haemochrome from the isolated haemin had its maximum at 552 nm, between that of protohaemin IX and haematohaemin. The test of Fischer & Gibian (1941) for free vinyl functions gave a positive result for both the *Crithidia* haemin and the haemoprotein.

The R_F values in the chromatography system 1 of Chu & Chu (1955) of haemin from *Crithidia* cytochrome, haematohaemin and protohaemin IX were 0.70, 0.87 and 0.40 respectively.

The amino acid composition of the protein per iron atom was: Asp₁₀, Thr₄, Ser₄, Glu₉, Pro₉, Gly₁₄, Ala₁₃, Val₇, Met₂, Ile₄, Leu₇, Tyr₃, Phe₅, His₂, Lys₁₀, Arg₄, Me₃Lys₁₋₂.

Three separate determinations of cysteic acid after oxidation of the haem-free protein gave

consistent values of 1.0–1.1 mol/mol. After carb-oxy-methylation 0.85 mol of carboxymethyl-cysteine/mol was found.

In the initial stages of sequence work a tryptic peptide has been isolated whose partially determined amino acid sequence is:

Ala-Ala-Gln-Cys-His(Thr,Gly,Ala)Lys

The sequence of the haem-binding region of eukaryotic cytochromes c follows the pattern:

Cys-X-Y-Cys-His

T. Meyer & M. A. Cusanovich (in preparation) have commented on the anomalous pyridine haemochromes of *Euglena* cytochrome 558 and *C. fasciculata* cytochrome 555 (Hill & White, 1968; Kusel, Suriano & Weber, 1969), which both have the 553 nm peak. They suggest the possibility of a 2(4)-vinyl-4(2)-ethyl-substituted haem and the substitution of a non-binding amino acid for one of the two haem-binding cysteine residues.

The results presented here suggest that the *C. oncopelti* cytochrome is of the same type and call into question the invariance of the covalent haem-binding in c-type cytochromes and parts of the definition of a cytochrome c.

G.P. thanks Dr A. P. Ryle and Dr J. L. Leaver for invaluable advice and encouragement, and we both thank Dr R. P. Ambler for his guidance and interest, especially in the latter stages of this work.

- Chu, T. C. & Chu, E. J. (1955). *J. biol. Chem.* **212**, 1.
 Fischer, H. & Gibian, H. (1941). *Justus Liebigs Annln Chem.* **548**, 183.
 Hill, G. C. & White, D. C. (1968). *J. Bact.* **95**, 2151.
 Kusel, J. P., Suriano, J. R. & Weber, M. M. (1969). *Archs Biochem. Biophys.* **133**, 293.
 Sletten, K., Dus, K., Klerk, H. & Kamen, M. D. (1968). *J. biol. Chem.* **243**, 5492.

The Metabolism *in vitro* of [ureyl-¹⁴C]-Tolbutamide by Microsomal Preparations from Rat and Human Liver

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The colorimetric methods commonly used to follow the rates of metabolism *in vitro* of various drugs are subject to error from high blanks, which arise from the presence in tissue fractions of interfering natural substances. For example, the assay of formaldehyde (commonly used to follow rates of metabolism of *N*-ethylmorphine, codeine, morphine, aminopyrine and many other drugs) is affected in this way. The use of radioactive drugs eliminates this problem, and also the problem of the lack of