formed into albumin. These results are almost identical with the data previously reported for rat liver[14].

The total immunoreactive albumin was 1.5-4% of the total homogenate protein, greater than the value reported for rat liver[13]. Since the human liver cannot be perfused prior to incubation, incomplete removal of serum from liver slices could account for an increased amount of serum albumin in the homogenate. The radioactive proalbumin (fractions 47-54 in fig. 1b) was completely separated from the albumin peak by a 2nd DEAE-chromatography. The purification procedure of 1 experiment is given in the table. During the purification the radioactivity per mg proalbumin decreased from 2180 to 1670 cpm/mg. In contrast, the peak coeluting with serum albumin contained only 620 cpm/mg (450 cpm/mg for the 2nd experiment). In 4 of the 6 samples, incubated for 30 min, the purification procedure for proalbumin was modified. From the acetone-dried powder the immunoreactive albumin was precipitated with specific antiserum and the dissolved immunoprecipitate submitted to Sephadex- and two subsequent DEAE-chromatographies. The yield of proalbumin was 15-90 µg with the radioactivity ranging from 1378 to 4033 cpm per mg. This small amount, again probably due to the long period of ischemia of the liver, made it impossible to determine the N-terminal sequence. The reproducible elution pattern on DEAE-chromatography, however, strongly suggests that human albumin is initially synthesized in a precursor form and is later transformed into serum albumin. The circulating albumin variant Christchurch found recently in a New Zealand family[15] indicates that the amino acid sequence of the N-terminal of human proalbumin is very similar to that of rat proalbumin.

1 This work was supported by grants from the Deutsche Forschungsgemeinschaft, the National Institutes of Health (CA 123896, AM 191241, CA 142940), and the Hochschulstiftung an der Universität Bern.
2 We gratefully acknowledge the excellent technical assistance of Ms Kathleen O'Laughlin and Ms Edith Junker-Riesen.
3 G. Schreiber, H. Dryburgh, K. Weigand, M. Schreiber, J. Witt and G. Howlett, Archs Biochem. Biophys., in press (1981).
4 J.H. Russell and D.M. Geller, J. biol. Chem. *250*, 3409 (1975).
5 J. Urban, A.S. Inglis, K. Edwards and G. Schreiber, Biochem. biophys. Res. Commun. *61*, 494 (1974).
6 J.E. Patterson and D.M. Geller, Biochem. biophys. Res. Commun. *74*, 1220 (1977).
7 K. Weigand, E. Alpert, H. Keutmann and K.J. Isselbacher, Comp. Biochem. Physiol. *67B*, 115 (1980).
8 K. Weigand, Klin. Wschr. *55*, 295 (1977).
9 G. Schreiber, H.-M. Rotermund, H. Maeno, K. Weigand and R. Lesch, Eur. J. Biochem. *10*, 355 (1969).
10 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. biol. Chem. *193*, 265 (1951).
11 C.B. Laurell, Analyt. Biochem. *15*, 45 (1966).
12 R.J. Mans, G.D. Novelli, Archs Biochem. Biophys. *94*, 48 (1961).
13 J. Urban, M. Chelladurai, A. Millership and G. Schreiber, Eur. J. Biochem. *67*, 477 (1976).
14 J. Urban and G. Schreiber, Biochem. biophys. Res. Commun. *64*, 778 (1975).
15 S.O. Brennan and R.W. Carrell, Nature *274*, 908 (1978).

An in vitro kinetic study of the mixed inhibition of honeybee hemolymph PNP-α-D-glucosidase by sucrose[1]

M. Bounias and M.R.J. Morgan

Biochemistry Laboratory, I.N.R.A., Avignon Research Center, F-84140 Montfavet (France), and Department of Biological Sciences, University of Lancaster, Lancaster (England), 5 March 1981

Summary. Sucrose acts in vitro as a mixed inhibitor of (V + K + n) type towards honeybee hemolymph PNP-α-D-glucosidase activity. Between the stages of emergence and foraging, there is little change in the effect of the inhibitor on V_M (f_i = from 1.31 to 1.35 respectively) or n (f_i = from 1.09 to 1.07) but K is more markedly affected (f_i^{-1} = from 1.17 to 1.87). These observations reflect the decrease of K_i from 277 to 98 mM and of I_{50} from 154 to 111 mM, but K_i' scarcely alters during development (from 477 to 425 mM). These inhibitory effects of sucrose are intermediate between those previously reported for trehalose and glucose.

In vertebrates, and in microorganisms, the biosynthesis of some enzymes can be adapted to nutritional or metabolic demand[2,3]. These induction phenomena commonly concern the enzymes of sugar metabolism and particularly those of the glycosidase group[4,5]. Some evidence has been presented previously[6,7] suggesting the induction of honeybee hemolymph α-glucosidase by trehalose. In beekeeping, it is common practice to feed honeybees with sucrose syrups at certain periods of the year. We have therefore investigated the influence of sucrose feeds on the glycemia of honeybees and on the induction of their hemolymph α-glucosidase activity (Bounias and Morgan, unpublished). As an essential preliminary to this work, it was necessary to determine any direct effects of sucrose on the kinetics of the α-glucosidase in order to be able to distinguish these from true induction effects.

Materials and methods. Since the induction experiments were to be conducted over a period of 16 days, the present study encompassed the 2 extreme developmental stages represented by this interval, so as to compare young emerging worker bees, *Apis mellifica mellifica* L., with foraging adults (16-day-old bees will soon commence foraging). The kinetics of α-glucosidase were investigated as previously described[8] using pNP-α-D-glucoside as a specific artificial substrate.

Hemolymph was removed by puncture and centrifuged as previously described[8,9] then diluted by 40 times in the reaction medium. The major α-glucosidase fraction (over 85%) was shown to be cooperative at prenymphal stages[9]; its sp. act. is 0.4 mM · min^{-1}/µg protein. The kinetic contribution of the minor fraction appears as non-significant (less than 1%) when evaluated according to algebraic methods suited for 2 superimposed kinetics[14]. The main parameters (V_M = maximum velocity; K = affinity constant; n = Hill coefficient) were determined according to a non-iterative algebraic method[9] applicable to the general case of the Hill equation, and in Michaelian cases (where n = 1 and K becomes K_m) also according to the Augustinsson/Hofstee plot[10]. The dose/response relationships for sucrose as inhibitor (I) were interpreted according to the Dixon plot[11] and

to the general equation of Chou[12]: $(V_0/V_i)-1=(I/I_{50})^{ni}$ applicable to cases where the Hill coefficient n_i for the binding of the inhibitor to the enzyme may differ from 1, I_{50} being the inhibitor concentration giving a 50% reduction in the initial velocity.

Results. A) Emerging bees. The saturation curves, represented as Augustinsson plots, are illustrated in figure 1a and b. Only the control series exhibits Michaelian kinetics; the linear regression for 7 points gives a correlation coefficient $r=0.997$, $K_m=4.64$ mM, $V_M=10.23$ $\mu M \cdot min^{-1} \cdot \mu l^{-1}$. The parameters calculated from the general equation[9] are given in the upper part of the table.

The inhibition results, represented as Dixon plots, are depicted in figure 1c; for values of (I) > 150 mM, there is unacceptable departure from linearity. Analysis of the data for (S) = 1 mM according to the logarithmic form of the Chou[12] equation gives: $r=0.964$ (for $N=8$ points); $n_i=0.656$; $I_{50}=154.4$ mM. For values of (I) < 150 mM, the Dixon plots reveal a mixed type of inhibition and give a triple intersection corresponding to $K_i=248.1\pm22.5$ (SD) mM.

B) Foraging bees. The Augustinsson plot (fig. 2a and b) is again linear only for the control series: $r=0.998$ ($N=10$ points); $K_m=3.19$ mM; $V_M=839.7$ $\mu M \cdot min^{-1} \cdot \mu l^{-1}$. Estimates of the kinetic parameters derived from the more rigorous method[9] are indicated in the lower part of the table. Dixon plots (fig. 2c) for the inhibited series are more nearly linear than those for the emerging bees, with a triple intersection point signifying mixed inhibition and corresponding to $K_i=103.9\pm25.4$ mM. Fitting the Chou equation to the data for (S) = 1 mM gives: $r=0.994$ ($N=7$ points); $n_i=1.074$; $I_{50}=111.0$ mM.

Discussion. For the control series, foraging bees display much higher values of V_M and somewhat lower values of K_m than the emerging bees, in agreement with previous results[8] for other colonies. At both stages of development, in vitro inhibition by sucrose decreases the Hill coefficient n, an effect analogous to that found for glucose inhibition[8]. The inhibition factors f_i for n and V_M are similar for both stages, but K (and hence V_M/K) is more markedly affected at the foraging stage. These results are best interpreted in terms of a mixed inhibition mechanism (type V + K + n) in which the binding of the inhibitor sucrose to the enzyme and to the enzyme-substrate complex is characterized by the dissociation constants K_i and K'_i quoted in the table. The values of K_i reveal rather low enzyme-inhibitor affinity, particularly in emerging bees, and the affinity is yet further reduced in the ES complex to similarly low levels for both stages, as shown by the agreement in K'_i values. In spite of these low values for the inhibitor affinity, inhibition by sucrose may still be of considerable physiological significance since the hemolymph concentration of this disaccharide can reach levels as high as 100 or even 200 mM (Bounias & Morgan, unpublished). The Hill coefficient n_i for the binding of the inhibitor to the enzyme is well below 1 for emerging bees and slightly above 1 for foraging bees, reflecting the frequently non-Michaelian tendencies of the enzyme[8, 13].

The behaviour of sucrose as a mixed (V + K + n) inhibitor of honeybee hemolymph pNP-α-D-glucosidase differs somewhat from that of trehalose which gives either (V + K) type inhibition[13] or almost pure (n) type effects[14] with higher values of I_{50} and n_i for foraging bees than for emerging bees. Sucrose also differs from glucose which is a retroactive (K + n) inhibitor of the enzyme[8]. Since both

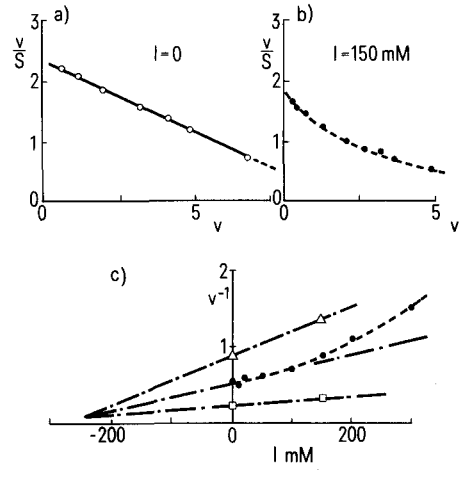

Figure 1. Kinetic effects of sucrose in vitro on the hemolymph PNP-α-D-glucosidase activity of emerging bees: Augustinsson plots for *a* control series, *b* sucrose-inhibited series; *c* Dixon plots at 3 substrate concentrations (S) = 0.5 mM (△), (S) = 1 mM (●), (S) = 4 mM (□).

Modification of the kinetic parameters of honeybee hemolymph PNP-α-D-glucosidase activity by sucrose in vitro. V_M is expressed in $\mu M \cdot min^{-1} \cdot \mu l^{-1}$; K_m, K_i and K'_i in mM. The values in parentheses are standard deviations for N determinations

	Control (I) = 0	Sucrose-inhibited (I) = 150 mM	f_i	K_i	K'_i
Emerging bees					
N	5	6			
V_M	10.20(0.87)	7.76(1.31)	1.314		477.0
K or K_m	4.44(0.46)	5.21(1.04)	1.173^{-1}		
V_M/K	2.297	1.489	1.542	276.6	
n	0.980(0.029)	0.898(0.045)	1.091		
Foraging bees					
N	7	7			
V_M	830.8(79.6)	613.9(84.6)	1.353		424.5
K or K_m	3.10(0.39)	5.79(0.93)	1.868^{-1}		
V_M/K	268.0	106.0	2.528	98.19	
n	0.997(0.045)	0.936(0.035)	1.065		

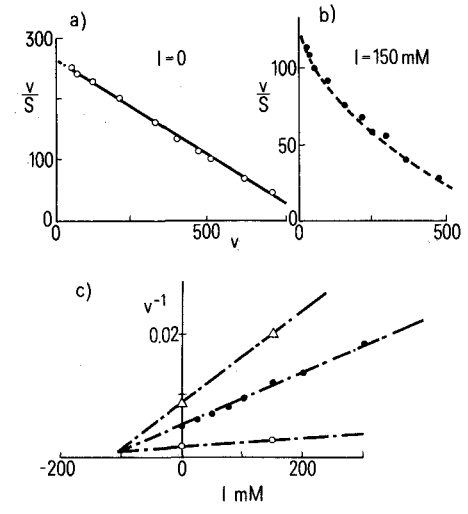

Figure 2. Kinetic effects of sucrose in vitro on the hemolymph PNP-α-D-glucosidase activity of foraging bees: Augustinsson plots for *a* control series, *b* sucrose-inhibited series; *c* Dixon plots at 3 substrate concentrations (S) = 0.5 mM (△), (S) = 1 mM (●), (S) = 9 mM (□).

sucrose and trehalose depress the velocity of the enzyme-catalyzed reaction, their effects on the kinetic parameters of the enzyme should not cause any confusion in the interpretation of the induction experiments which test for a substrate-induced increase of the enzyme activity resulting from de novo protein biosynthesis.

1 Acknowledgments. Particular appreciation is due to Mrs C. Joliet for her meticulous technical assistance, and to Mr R. Paris for his care as beekeeper. Dr M.R.J. Morgan is grateful for financial support from The Leverhulme Trust, The Royal Society, I.C.I. Plant Protection and Shell International Petroleum.

2 P. Desnuelle, Revue fr. Étud. clin. biol. *8*, 494 (1963).
3 J.M. Robert-Baudouy, R.C. Portalier and F.R. Stoeber, Eur. J. Biochem. *43*, 1 (1974).
4 W.M. Fitch and I.L. Chaikoff, J. biol. Chem. *235*, 554 (1960).
5 P. Desnuelle, Proc. 2nd Int. Coll. 'Automatisation and Prospective Biology', Pont-à-Mousson 1972, p. 22. Karger, Basel 1973.
6 M. Bounias, Bull. Soc. zool. Fr. *102*, 294 (1977).
7 M. Bounias, Comp. Biochem. Physiol. *66B*, 335 (1980).
8 M. Bounias, Experientia *36*, 157 (1980).
9 M. Bounias, Comp. Biochem. Physiol. *63B*, 407 (1979).
10 K.-B. Augustinsson, Acta physiol. scand. *15*, suppl. 52 (1948).
11 M. Dixon, Biochem. J. *55*, 170 (1953).
12 T.-C. Chou, J. theor. Biol. *59*, 253 (1976).
13 M. Bounias and M.R.J. Morgan, C.r. Soc. Biol. *175*, 12-20 (1981).
14 M. Bounias and M.R.J. Morgan, C.r. Acad. Sci., Paris, Ser. III 883 (1981).

The brachymorphic mutation of mice and altered developmental patterns of limb bud 3′:5′ cyclic adenosine monophosphate[1]

D. Hindin and R.P. Erickson

Department of Human Genetics, Box 015, University of Michigan Medical School, Ann Arbor (Michigan 48109, USA), 8 December 1980

Summary. Concentrations of cyclic AMP (cAMP) were determined in paired fore and hind limbs from day 12-16 of development in murine fetuses homozygous for the brachymorphic *(bm)* mutation and normal controls. A developmental rise in cAMP occurred 1 day earlier in *bm/bm* than in +/+ hind limbs and cAMP was higher in day-13 *bm/bm* than in +/+ fore limbs. Since cAMP is well documented to stimulate chondrogenic differentiation, premature cartilage determination secondary to altered levels of cAMP could play a role in *bm/bm* short-limbed dwarfism.

Homozygous brachymorphic mice *(bm/bm)* are characterized by a disproportionate shortening in the length of all bones where growth occurs by cartilage proliferation[2]. Studies by Orkin and co-workers[3] of epiphyseal cartilage indicated the presence of altered proteoglycans (reduced in size and number) in *bm/bm*. The majority of the proteoglycans appeared as a network of thin filaments associated with collagen in *bm/bm*, whereas the normal appearance consisted of polygonal matrix granules. On the other hand, the collagen in the extracellular matrix surrounding the cartilage chondrocytes appeared normal. In addition, *bm/bm* glycosaminoglycans (GAG) were identified as a type of chondroitin sulfate that was undersulfated[4]. Greene et al.[5] found alterations in GAG synthesis to be most severe in the proliferative zone of the epiphyseal growth plate of *bm/bm* limbs. In contrast, epiphyseal protein was found to be normal in *bm/bm*.

This under-sulfation in *bm/bm* has been hypothesized to be due to a deficiency in the enzymatic conversion of adenosine 5′-phosphosulfate (APS) to 3′-phosphoadenosine 5′-phosphosulfate (PAPS), a general sulfate donor[6]. In particular, the defect in *bm/bm* cartilage was related to a 93% decrease in levels of APS kinase. These observations were made on neonatal *bm/bm* mice compared to the +/+ mice from the congenic control strain (C57BL/6J) - dosage was not shown in heterozygotes. Interestingly, the metabolically-related compound, cyclic AMP (cAMP) was found to be altered in palatal shelves of *bm/bm* mice which were studied because of the high susceptibility to cortisone-induced cleft palate determined by homozygosity at the brachymorphic locus[7]. Since alterations in cAMP levels in limb mesenchyme appear to trigger chondrogenesis[8,9], we have studied concentrations of cAMP in developing limb buds of *bm/bm* and syngeneic normal (+/+) fetuses.

Materials and methods. Brachymorphic mice were obtained from K.S. Brown, N.I.D.R., N.I.H., and a colony was established. Pregnant *bm/bm* and C57BL/6J females (mated to their respective males) were sacrificed by decapitation at 12-16 days of gestation. Day 0 (zero) was determined by the discovery of a vaginal plug in the morning. Embryos removed from these females (110 *bm/bm* and 85 C57BL/6J fetuses were used in total; they were unevenly distributed among days) were double checked for the specific day of development using external characteristics as described in Theiler[10]. After sacrificing the mother, the uterus was immediately dissected out and placed in a warmed, high glucose media (0.2 M glucose, 0.05 M NaCl, 0.02 M Na_2HPO_4, and 0.0026 M KH_2PO_4). Each embryo was individually removed from the uterus and the 2 front and 2 back limb buds dissected off while in the high glucose media. The removed limb buds were placed in a test tube with 300 µl H_2O and immediately placed in a boiling water bath for 10 min. This method of deproteinizing tissue has been found to be superior to perchloric and trichloracetic acid precipitations[11]. After cooling on ice, the 2 samples from each embryo (front and back limb bud aliquots) in the litter were then centrifuged at 1300×g for 15 min at 4 °C. 200 µl from each supernatant was aliquoted, divided into 2×100 µl duplicates and stored for cAMP assay. The remaining 100 µl liquid and limb pellets were saved for protein determination by the method of Lowry.

cAMP levels were measured by a modification of the radioimmunoassay of Steiner et al.[12] using a commercially available kit (New England Nuclear) and expressed in pmol cAMP per mg of limb bud protein.

Results and discussion. Hind limb concentrations of cAMP increased from day 12 to day 14 in *bm/bm* fetuses and then decreased to day 16 (fig. A). Normal hind limbs showed a nearly identical developmental pattern which was delayed by 1 day, however (fig. A). Fore limb concentrations of cAMP were nearly identical to hind limb concentrations in normal fetuses (fig. B) while in *bm/bm* fetuses, they were