Molecular weight of alanine aminotransferase from soluble and mitochondrial fractions (both after DEAE-cellulose chromatography) of locust flight muscle

Fraction	Ve/Vo	Molecular weight (daltons)	Multiplication factor
Soluble[a]			
S_I	1.59	63,000	1
S_{II-B} minor	1.34	139,500	2
S_{II-A} major	1.15	257,000	4
Mitochondrial			
M_B major	1.39	119,000	2
M_A minor	1.17	241,000	4

A calibrated column, 107 × 1.3 cm, was fitted to the regression equation, log M = 7.00452 — 1.3865. (Ve/Vo), where M is molecular weight, Ve and Vo are elution and void volumes respectively in milliliters.

[a] Soluble enzyme peak I and peak II were chromatographed.

differences at the quaternary level[10]. That the species we observed in the present investigations were indeed the isomeric forms of the enzyme finds support in our results on the molecular weight determination by gel chromatography on Sephadex G-200.

Our observations (Table and Figure 2) on the flight muscle soluble enzyme I and II indicated that these forms are monomer and tetramer, while the mitochondrial enzyme is a dimer. Further, the soluble enzyme peak II was associated with a dimeric minor enzyme protein which was not completely separated from the main (Figure 2, S). Similarly, mitochondrial fraction also showed incomplete chromatographic separation of the minor tetrameric enzyme protein from the main (Figure 2, M). This clearly suggests the occurrence of the dimer-tetramer complexes of enzyme in the cytosol as well as in the mitochondria. Although, the physiological significance of these structural subunits is not clear, it may nevertheless be added that they may play an important role similar to glycerophosphate or malate-oxaloacetate cycle in the proper maintenance of NAD/NADH ratio[11].

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Effects of Starvation and Ca^{++} on Glucose-Induced Accumulation of Cyclic 3′,5′-AMP in Pancreatic Islets[1]

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Summary. Exposure to glucose in the presence of 3-isobutyl-1-methylxanthine leads to accumulation of cAMP in islets microdissected from *ob/ob*-mice. This process is dependent on extracellular Ca^{++} but differs markedly from the glucose action on insulin release in the same in vitro system in disappearing after 18 h of starvation.

In analogy with the Sutherland hypothesis of hormone action through a second messenger, glucose initiation of insulin release has been supposed to occur as a result of receptor interaction leading to the formation of cyclic adenosine 3′,5′-monophosphate (cAMP)[2–5]. Although this concept has been challenged in several reports[6–11], it seems likely that accumulation of cAMP contributes to the self-potentiating action of glucose as an insulin secretagogue[9–11].

There has been a great deal of controversy as to whether exposure to glucose actually leads to accumulation of cAMP in the pancreatic islets[3–7,9–11]. Recent studies in this laboratory showed that perfusion with 20 mM glucose results in a significant increase of cAMP in islets microdissected from fed obese-hyperglycaemic mice (genotype *ob/ob*), provided that they are simultaneously exposed to a potent phosphodiesterase inhibitor[11]. The present study adds to the characterization of the glucose-induced accumulation of cAMP in these β-cell-rich pancreatic islets by demonstrating its dependence on the nutritional status of the animal and the presence of extracellular Ca^{++}.

Materials and methods. Female 7 month old *ob/ob*-mice, taken from a non-inbred colony, were used as the source of pancreatic islets containing more than 90% β-cells[12]. The animals had free access to water and were either allowed free access to food, or starved for 18 h before being killed by decapitation. Fresh pancreatic islets were microdissected free-hand and incubated at 37 °C with different concentrations of Ca^{++} in Krebs-Ringer bicarbonate medium containing 1 mg/ml albumin and equilibrated with O_2 + CO_2 (95:5). After 40 min of preliminary incubation, the amounts of insulin released were measured during 60 min of further incubation in medium containing 3 or 20 mM glucose and 1 mM 3-isobutyl-1-methylxanthine (IBMX). When analyzing the

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islets from the fed animals, 0.5 m*M* ethylene-glycol-*bis*-(aminoethylether)-tetra-acetic acid (EGTA) was included in the media lacking Ca^{++}. After incubation, the islets were freeze-dried and weighed as previously described[13].

The medium content of insulin was measured radioimmunologically using crystalline mouse insulin as a reference. Free and antibody-bound insulin were separated by precipitation with ethanol. For determination of cAMP, perchloric acid extracts were made from the freeze-dried islets, and in some cases, also from the incubation media. These extracts were purified by ion exchange chromatography and cAMP was measured by radioimmunoassay[11]. Statistical differences were calculated from the mean differences between paired test and control incubations in series of repeated experiments.

Results. The amounts of insulin released at different Ca^{++} concentrations in the presence of the phosphodiesterase inhibitor IBMX are shown in Table I. Raising the glucose concentration from 3 to 20 m*M* resulted in a significant stimulation of insulin release only when extracellular Ca^{++} was present. Further stimulation of insulin release by glucose beyond that noted in the ordinary KRB medium was not seen after including twice as much Ca^{++} (5.12 m*M*) in the incubation medium. Fasting resulted in a considerable reduction of the rate of insulin release recorded at 3 m*M* glucose. However, the nutritional status did not influence the secretory rate reached when the islets were stimulated with 20 m*M* glucose.

It is evident from Table II that higher levels of cAMP occurred in the islets microdissected from the mice allowed free access to food. In the presence of 3 m*M* glucose, the Ca^{++} concentration of the incubation medium did not influence the islet content of cAMP. Addition of 20 m*M* glucose to the incubation medium resulted in an increased islet content of cAMP when extracellular Ca^{++} was present. This effect of glucose on cAMP was statistically significant only with regard to the islets from fed animals and had no obvious counterpart for the cAMP found in the incubation medium.

Discussion. Whereas starvation for 48 h has been reported to lower the cAMP content of non-incubated islets isolated from rats[14], no such effects were noted with islets from normal, non-obese mice[10]. Prolonged withdrawal of food also did not reduce the cAMP levels recorded after incubating mouse islets with low or high concentrations of glucose in the presence of 1 m*M* of the potent phosphodiesterase inhibitor IBMX[10]. In contrast, the short period of starvation employed in the present study caused a marked depression of cAMP when the β-cell-rich *ob/ob*-mouse islets were incubated with the same concentration of IBMX. It was also evident that withdrawal of food resulted in a disappearance of the glucose-induced accumulation of cAMP seen in the presence of the phosphodiesterase inhibitor. The latter finding lends strong support to the hypothesis of CAPITO and HEDESKOV[10] that the process by which glucose leads to accumulation of cAMP in the pancreatic islets[3–7, 9–11] is dependent on the nutritional status of the animals. The fact that the effect of starvation on the glucose accumulation of cAMP was not accompanied by any reduction of glucose-stimulated insulin release adds to the previous observations indicating fundamental differences between the cAMP-promoting and insulin-releasing activities of glucose[6, 7, 9–11]. It is worthy of note that there was no impairment of the glucose-induced insulin release in the presence of IBMX after removal of food, since previous studies have indicated a suppressant effect of fasting after exposing islets from *ob/ob*-mice to theophylline[15]. This discrepancy may be attributed to the fact that IBMX is a more potent phosphodiesterase inhibitor.

Adenylate cyclase activity has been found to depend on Ca^{++} in other types of cells[16, 17], but there are also reports that Ca^{++} can inhibit the formation of cAMP[18–21]. The latter type of effect is exemplified from the increased levels of cAMP noted in cerebral cortical slices after omission of Ca^{++} from the incubation medium[22]. It has been demonstrated that withdrawal of Ca^{++} from a Krebs-Ringer bicarbonate medium containing high concentrations of glucose and phosphodiesterase inhibitor results in a slight depression of the cAMP levels in islets microdissected from *ob/ob*-mice starved overnight[23]. The present study extends those observations in showing that Ca^{++} is a prerequisite for the glucose-induced accumulation of cAMP rather than influencing the level of this nucleotide at a non-stimulating glucose concentra-

Table I. Amounts of insulin released

Nutritional status	Ca^{++} (m*M*)	Insulin release (ng/μg dry islet) Glucose (3 m*M*)	Glucose (20 m*M*)	Difference
Starved	0	0.96 ± 0.21	2.74 ± 0.91	+ 1.78 ± 0.87
Starved	2.56	1.77 ± 0.23	26.65 ± 2.65	+24.89 ± 2.53[b]
Starved	5.12	1.60 ± 0.35	25.02 ± 3.76	+23.43 ± 3.58[b]
Fed	0	3.34 ± 0.94	4.37 ± 1.88	+ 1.03 ± 1.14
Fed	2.56	12.27 ± 2.64	27.78 ± 3.46	+15.50 ± 2.02[b]
Fed	5.12	10.51 ± 2.07	26.46 ± 5.48	+15.96 ± 4.34[a]

Islets were microdissected from starved and fed *ob/ob*-mice and incubated for 60 min with 3 or 20 m*M* glucose in the presence of different concentrations of Ca^{++}. Mean values ± SEM for 7–8 experiments. [a] $p < 0.01$; [b] $p < 0.001$.

Table II. Islet contents of cAMP and the amount of cAMP released into the medium

Nutritional status	Ca (m*M*)	Islet cAMP (fmoles/μg dry islet) Glucose (3 m*M*)	Glucose (20 m*M*)	Difference
Starved	0	16.9 ± 2.0	20.7 ± 4.4	+ 3.8 ± 4.3
Starved	2.56	21.0 ± 2.8	25.1 ± 3.3	+ 4.1 ± 5.1
Starved	5.12	18.0 ± 0.7	29.1 ± 4.5	+11.1 ± 4.6
Fed	0	29.5 ± 6.3	35.8 ± 5.3	+ 6.3 ± 3.4
Fed	2.56	30.5 ± 3.5	52.3 ± 7.1	+21.8 ± 5.3[a]
Fed	5.12	32.9 ± 4.8	59.5 ± 7.9	+26.6 ± 7.2[a]
		Medium cAMP (fmoles/μg dry islet)		
Fed	0	39.8 ± 6.2	33.4 ± 4.6	− 6.3 ± 6.9
Fed	2.56	28.0 ± 3.0	30.7 ± 2.9	+ 2.7 ± 1.9
Fed	5.12	28.8 ± 4.0	32.4 ± 5.2	+ 3.6 ± 4.2

Microdissected islets were incubated for 60 min with 3 or 20 m*M* glucose in the presence of different concentrations of Ca^{++}. The islets are those described in Table I. Mean values ± SEM for 7–8 experiments[a]. $p < 0.01$.

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tion. Recent reports also suggest that Ca^{++} is a prerequisite for glucose-induced accumulation of cAMP in rat islets[5, 24].

Calcium ions may stimulate the accumulation of cAMP in pancreatic islets exposed to high concentrations of glucose in several ways. One possibility is that Ca^{++} inhibits the degradation of cAMP. This seems unlikely in view of the observation that glucose-induced accumulation of cAMP requires that the phosphodiesterase activity be inhibited in the present islet preparation[11]. Furthermore, Ca^{++} has not been found to affect the phosphodiesterase activity in mouse islets[25, 26]. Omission of extracellular Ca^{++} does not significantly reduce the islet oxidation of glucose at glucose concentrations below 5 mM[27], i.e. the concentration range in which ATP is critically dependent on glucose[28]. Therefore the promoting effect of Ca^{++} on cAMP accumulation might reflect changes in the adenylate cyclase activity rather than being the result of increased β-cell levels of ATP.

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Phylogenetic Position of the American *Timarcha* Latr. (*Coleoptera*, Chrysomelidae) Based on Chromosomal Data

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Summary. The chromosomal analysis of *T. intricata* Hald. has shown a diploid complement of 44 chromosomes, the highest number found in *Timarcha* and clearly different from those of the taxa previously studied. This complement favours a derivative origin of the *Americanotimarcha* subgenus from a hypothetical ancestral species of 20 chromosomes. The implications of this karyotype are also discussed with some morphological, biogeographical and ecological points of view and observations.

Since 1968, the karyological analyses carried out on 31 taxa of the genus *Timarcha* Latreille have revealed a rather wide range of variation in chromosome numbers from $2n = 20$ to $2n = 30$[2–5]. By using morphological criteria, the genus appears as sharply homogeneous; thus these unsuspected karyological differences have provided valuable tools to establish the primitiveness of the genus and have thrown light on evolutionary lineages. The karyological results were in a good agreement with some others obtained in various aspects of taxonomic interest[6].

Most *Timarcha* have a diploid complement of 20 chromosomes, a number which has been considered as the primitive among the *Coleoptera polyphaga*[7]. All taxa cytologically examined belong to the subgenus *Timarcha s. str.* (including in this sense the subgenus *Timarchostoma* Mots.), but representatives of the two other subgenera, *Metallotimarcha* Mots. and *Americanotimarcha* Jolivet, were not chromosomically analyzed.

According to several characters of external morphology and male genitalia structure, the *Metallotimarcha* and the *Americanotimarcha* are generally considered as the most ancient *Timarcha*. This aspect is presumably related with the relict geographical distribution of both subgenera, the *Metallotimarcha* in hercynian mountains of Central Europe and in the Caucasus, and the *Americanotimarcha* in western coast of North America from Vancouver (Canada) to northern California[8, 9]. However, ecological observations on the *Americanotimarcha* suggest a derivative origin of this subgenus from the ancestral generic source, since the two species of *Americanotimarcha* feed on Roseaceae while most of the other *Timarcha* feed, actual or potentially, on Rubiaceae though they may be secondarily adapted to plants of other families[8, 9]. From this point it seemed particularly interesting to know some cytological data on the *Americanotimarcha* to determine its phylogenetic position within the genus.

Material and method. A small sample of male living individuals of *T.* (*Americanotimarcha*) *intricata* Hald. were air-mailed in June 1974 from Oregon to Barcelona and immediately studied on arrival. The chromosome analysis was performed on meiotic and mitotic metaphases of spermatogonial cells by aceto-orcein squash preparations. Some microphotographs were also taken of the best metaphase spreads which complemented the microscopic observations.

Results. Two individuals were cytologically examined and both showed a diploid complement of 44 chromosomes. The karyotype of this species is constituted by chromosomes of small size, mostly acrocentrics (Figure 1). 22 rod/round shaped bivalents were easily recorded in

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