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PROPERTIES OF LACTOSE SYNTHETASE FROM MOUSE MAMMARY GLAND: ROLE OF A PROPOSED THIRD COMPONENT

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SUMMARY

1. The properties of lactose synthetase from mouse mammary tissue were investigated and found to resemble those of the cow and rat.

2. Lactose synthetase is composed of two subunits, A and B; both are required for lactose-synthetase activity. Gel filtration of partially purified homogenates of mouse mammary tissue on Sephadex G-100 reveals the molecular weight of B to be about 15 000. The A protein elutes as three peaks: one at the void volume, a second with proteins having a molecular weight of 100 000–130 000, and a third at 29 000.

3. Crude homogenates of lactose synthetase have different properties than partially purified preparations. When crude preparations of lactose synthetase are diluted the resulting activity is proportional to the extent of dilution; however, the dilution of purified preparations results in an exponential decrease in activity. Also, restoration of lactose-synthetase activity by recombination of A and B subunits follows different kinetics with the two types of preparations.

4. A third component, Z, has been proposed which in crude preparations has the property of preventing rapid dissociation of the AB complex. During purification, Z is lost and the A and B subunits then obey the laws of mass action and chemical equilibrium.

INTRODUCTION

Lactose synthetase (EC 2.4.1.22) catalyses the synthesis of lactose from uridine diphospho-D-galactose (UDPGal) and glucose and is composed of two subunits, both of which are required for activity[1]. One of the subunits (A) has *N*-acetyllactosamine (NAL) synthetase activity on its own (NAL synthetase transfers galactose from UDPGal to *N*-acetylglucosamine)[2]. The other subunit (B) is identical to the milk protein, α-lactalbumin[1].

Lactose synthetase has recently been isolated from cow's milk[1–3], and from

Abbreviations: UDPGal, uridine diphospho-D-galactose, NAL, *N*-acetyllactosamine.

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bovine mammary gland tissue[1]. Some investigations of lactose-synthetase activity[4] and properties[2,5] have been conducted using the rat.

Both subunits of lactose synthetase have been induced in cultures of mouse mammary tissue by addition of the hormones, insulin, hydrocortisone and prolactin[6,7] in a manner similar to the induction of other milk proteins[8,9].

Investigation of the properties of lactose synthetase from the mouse and especially the comparison of the properties of crude homogenates with partially purified preparations has led to the proposal of a third component of lactose synthetase. The third component has the property of stabilizing the complex of A and B subunits.

MATERIALS AND METHODS

Preparation of homogenates

Inguinal mammary glands of either pregnant nulliparous or lactating primparous BALB/C strain mice were placed in Hank's balanced salt solution (Grand Island Biological); lymph nodes and superficial connective tissues were discarded; and the glands were cut into about 10 pieces. All succeeding steps were carried out at 2–4°. The tissue (1.0–3.0 g) was then washed and homogenized in 10 ml of 0.02 M Tris–HCl (pH 7.5) 0.01 M $MgCl_2$ buffer (Tris–Mg^{2+} buffer) unless otherwise noted. A 30-ml, glass–teflon, motor-driven homogenizer was used; 4–8 strokes within 1 min. The crude homogenate was then poured through a column of loosely packed glass wool to remove large pieces of cellular debris. Homogenates of lactating tissue were freed of small molecules by gel filtration through a 1.5 cm × 25 cm column of Bio-Gel P-6 using Tris–Mg^{2+} buffer. The resulting homogenates were generally used without further fractionation since under these preparative conditions about 60% of the lactose-synthetase activity is removed from the supernatant by centrifugation for 10 min at 15 000 × *g*.

Enzyme assays

Lactose synthetase and NAL-synthetase activities were determined as in previous studies[6] by a modification of the method of BABAD AND HASSID[3]. A typical reaction mixture contained 2 μmoles of Tris–HCl (pH 7.5), 1 μmole $MgCl_2$, 0.5 μmoles $MnCl_2$, 0.15 μmoles ATP, 32.3 nmoles UDP-[$^{14}C_6$]galactose (24 000 disint./min, International Chemical and Nuclear), and either 3 μmoles glucose (for the determination of lactose-synthetase activity) or 0.45 μmoles of *N*-acetylglucosamine (for the determination of NAL-synthetase activity) in a final volume of 150 μl. The 400-μl plastic tubes containing the reaction mixture were incubated at either 37 or 40° for up to 30 min. The reaction rate was constant for 30 min as long as 15% conversion of UDPGal was not exceeded. Controls without the substrates, glucose or *N*-acetylglucosamine, were included to correct for non-specific hydrolysis of UDPGal. This non-specific hydrolysis was kept at a minimum by including ATP at an initial concentration of 1 mM. ATP at this concentration not only inhibits nucleotide pyrophosphatases but also gives twice as much lactose-synthetase activity as controls without ATP. In some early experiments UTP at 5 mM replaced ATP. The optimum concentration of Mn^{2+} was found to be 3 mM. The reaction was started by adding the radioactive UDPGal and stopped by placing the reaction mixture on top of an anion-

exchange column (consisting of about 1 ml of packed Bio-Rad AG1-X2 resin (Cl^- form) made up in distilled water in a glass-wool-plugged Pasteur pipette.) It was not possible to stop the reaction by lowering the temperature to 0° (see ref. 3) since the combined reaction rates of lactose synthetase and non-specific hydrolysis were only lowered to about 1/3 the rate measured at 37°. Lactose was eluted from the column with three 0.4-ml washes of distilled water. After the last wash, the remaining fluid was expelled by subjecting the column to gentle pressure from a Pasteur bulb. The washes containing the radioactive products were collected in a scintillator vial and counted in 10 ml of Bray's solution[10] using a Nuclear Chicago, Mark I, scintillation counter. Counting efficiency was between 70–75% and disint./min were calculated by the channels ratio method. In control experiments, [^{14}C]lactose was applied to the column and eluted as described above; average recovery was 98%. When non-specific hydrolysis of UDPGal was inhibited, 97% of the radioactive reaction products eluting through the column migrated like lactose when subjected to thin-layer chromatography (Gelman ITLC-type SG; NH_4OH–isopropanol (1:4, v/v)).

Tryptophan-synthetase activity was assayed according to the method of SMITH AND YANOFSKY[11].

Arginase, and ornithine transcarbamylase activities were assayed according to the methods of RIGHETTI, DE LUCA AND WOLF[12] and BROWN[13] respectively.

Protein was determined by the method of LOWRY *et al.*[14].

RESULTS

Decay of lactose synthetase activity

Lactose-synthetase activity in crude homogenates decays with a half-life of about 13 h. Fig. 1 illustrates the decay at 4° of both lactose-synthetase and NAL-synthetase (A subunit of lactose synthetase) activity in a crude mammary gland homogenate from a lactating mouse (15 days *post partum*). It is apparent that the activity of lactose synthetase (complex of A and B subunits) is decaying faster than

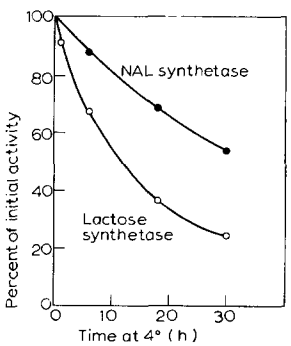

Fig. 1. Decay of NAL- and lactose-synthetase activities at 4°. Crude homogenates were prepared as in MATERIALS AND METHODS, including filtration through P-6 column, from a lactating mouse (15 days *post partum*). Enzyme activities were assayed according to standard procedures. (See MATERIALS AND METHODS.) Initially, NAL synthetase was able to transfer 175 counts/min of galactose from labelled UDPGal to *N*-acetylglucosamine each min at 37°; initially lactose synthetase was able to transfer 205 counts/min of galactose from labelled UDPGal to glucose each min at 37°.

TABLE I

EFFECT OF SONICATION AND HOMOGENIZATION ON NAL- AND LACTOSE-SYNTHETASE ACTIVITY

20 pieces (17–18 mg total) of mammary tissue from 14-day pregnant mice were sonicated in 1 ml of Tris–Mg^{2+} buffer for the indicated time at 0° with a Branson sonicator at a power output setting of 3. As a control, the same amount of tissue was homogenized in 1 ml of Tris–Mg^{2+} buffer in a 5 ml, glass–teflon, motor-driven homogenizer at 3° with 5 strokes within 30 sec. After sonication or homogenization the samples were filtered through a Pasteur pipette, which was plugged with glass wool, to remove unbroken clumps of cells and connective tissues. NAL and lactose synthetase were assayed using standard conditions (see MATERIALS AND METHODS); incubations were 15 min at 37°. Protein was determined by method of LOWRY *et al.*[14] except that after reaction and color development, samples and bovine serum albumin standards were extracted with diethyl ether to remove fat.

	Sonication					*Homogenization*
Number of 10-sec ultrasonic pulses	*1*	*2*	*3*	*4*	*5*	
Protein released (μg/ml)	17.6	33.8	33.2	36.5	36.4	23.5
NAL-synthetase activity (disint./min)	1517	4020	3622	3766	4527	2587
NAL-synthetase specific activity (disint./min per μg protein)	86	119	109	103	124	110
Relative NAL-synthetase activity (% of maximum)	69.4	95.9	87.9	83.1	100	88.7
Lactose synthetase activity (disint./min)	204	222	143	128	124	851
Lactose-synthetase specific activity (disint./min per μg protein)	11.6	6.6	4.3	3.5	3.2	36.2
Relative lactose-synthetase activity (% of maximum)	32.0	18.1	11.7	9.7	8.8	100

the activity of A alone. Because of the rapid loss of activity, crude homogenates were used within 1 or 2 h after preparation.

This decay phenomenon was further investigated by ascertaining the differential effect of sonication on the A subunit alone and on the AB complex (Table I). Sonication was performed in an ice bath with a Branson sonicator using the micro tip. Twenty pieces (17–18 mg) of 14-day pregnant mammary gland tissue were placed in 1 ml Tris–Mg^{2+} buffer; sonicated with up to five 10-sec pulses interspersed with 30 sec cooling periods. In a control experiment the same amount of tissue was homogenized in 1 ml of Tris–Mg^{2+} buffer according to standard techniques (see legend of Table I). 20 sec of sonication liberates nearly maximal NAL-synthetase activity and further sonication has little effect. In contrast, continued sonication causes a significant decline in lactose-synthetase activity. Homogenization, although not as effective as sonication at liberating protein, yields nearly maximal NAL synthetase specific activity and yields lactose synthetase with a specific activity 10 times greater than that seen after 50 sec of sonication. Thus, the activity of the AB complex is more sensitive to sonication than A alone.

Partial purification and molecular-weight determination of the A subunit of lactose synthetase

The A subunit was partially purified from lactating mammary glands according to previously published methods[6]. In brief, 10 ml of mammary-gland homogenate (200–300 mg of tissue per ml) were sonicated with four 10-sec pulses from a Branson sonicator at a setting of 2; $MnCl_2$ was added to 0.03 M and the homogenate was centrifuged for 10 min at 15 000 × *g* at 1°. Solid $(NH_4)_2SO_4$ was added to the resultant supernatant and that portion which precipitated between 35 and 85% saturation was resuspended in a minimal volume (1–2 ml) of Tris–Mg^{2+} buffer and subjected to gel filtration on a 1.4 cm × 120 cm column of Sephadex G-100 (Pharmacia). Tryptophan synthetase A protein (mol. wt. 29 000), bovine hemoglobin (mol. wt. 64 000) and phenol red (mol. wt. 354) were added as molecular weight markers in some

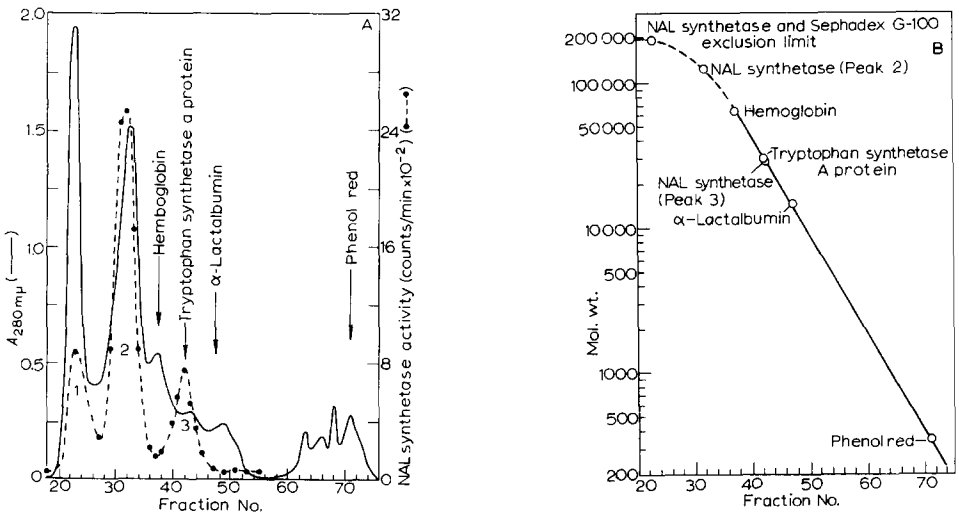

Fig. 2.A. Gel filtration of an $(NH_4)_2SO_4$-fractionated mouse mammary gland homogenate on Sephadex G-100. The homogenate was prepared as outlined in the text. Anterior inguinal mammary glands from three lactating (18 days *post partum*) mice were used in this preparation. The column (1.4 cm × 120 cm) was eluted with Tris–Mg^{2+} buffer; flow rate was 5.1 ml/h; each fraction contained 108 drops (2.8 ml). Enzyme activities were determined as described in MATERIALS AND METHODS. Hemoglobin and phenol red were localized by measuring the absorption at 412 and 560 nm respectively. Arrows indicate the fraction which contained the maximum concentration of the specified substance. B. Relationship between molecular weight and elution volume presented in A. Since the fractions were of constant volume (2.8 ml), fraction number is substituted for elution volume. The exclusion limit of Sephadex G-100 for globular proteins was assumed to be 200 000 mol. wt.

experiments. The A subunit was localized using the NAL-synthetase assay, and the B subunit (α-lactalbumin) was localized by combining an excess of the A subunit with aliquots from the other fractions and using the lactose-synthetase assay. Fig. 2A illustrates a typical elution profile. There are three activity peaks of NAL synthetase: Peak 1 eluted at the exclusion volume; Peak 2, comprising about 65% of the total activity, eluted with proteins having a molecular weight of about 100 000–130 000; and a third peak, which co-chromatographs with tryptophan synthetase A protein, had the highest specific activity of the three. These results tentatively

establish 29 000 as the minimal molecular weight for NAL synthetase, the A subunit of lactose synthetase. The other subunit, α-lactalbumin, has a molecular weight near 15 000 (Fig. 2B).

To ascertain whether the 29 000 molecular weight NAL-synthetase peak was generated from Peak 2 by either sonication or $(NH_4)_2SO_4$ precipitation Fractions 28–33 (from Sephadex G-100 column, Fig. 2A) were pooled, resonicated and reprecipitated as originally. They were then redissolved in Tris–Mg^{2+} buffer and passed through the Sephadex G-100 column for a second time. All the NAL synthetase migrated as a single peak which was coincident with the original Peak 2. The enzyme under that peak was then pooled, made 17 mM with respect to EDTA, precipitated with 85% $(NH_4)_2SO_4$ and redissolved in 20 mM Tris–HCl, 17 mM EDTA (pH 7.5). Meanwhile, the column was washed with 17 mM EDTA, followed by 20 mM Tris–HCl (pH 7.5) to remove all divalent ions from the column. The EDTA-treated enzyme was then eluted through the column with 20 mM Tris–HCl. NAL synthetase still migrated with the major protein peak and had a molecular weight of about 100 000–130 000. Thus, it seems unlikely that the enzyme in Peak 3 is generated from Peak 2 by a divalent metal ion association of the smaller molecular weight enzyme with some prevalent protein (such as a milk protein, like casein).

To investigate further whether the NAL synthetase from mammary tissue

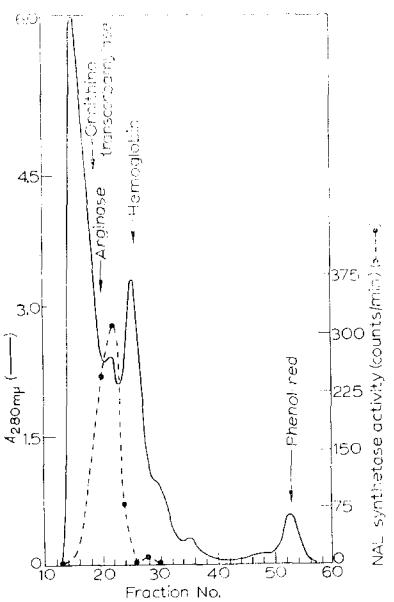

Fig. 3. Gel filtration on Sephadex G-100 of an $(NH_4)_2SO_4$-fractionated mouse liver homogenate. The liver homogenate was prepared in a manner identical to the preparation of mouse mammary gland NAL synthetase (see text). The livers (6.7 g) from four lactating mice (10–15 days *post partum*) were used. Only half of the resultant $(NH_4)_2SO_4$ precipitate was applied to the column. The column (1.5 cm × 85 cm) was eluted with Tris–Mg^{2+} buffer; flow rate, 6 ml/h; each fraction contained 108 drops (2.8 ml). NAL synthetase from mouse mammary gland shows peak activities in Fractions 16, 21, and 28 on this column. Arginase, ornithine transcarbamylase, and NAL synthetase were assayed as described in MATERIALS AND METHODS. Hemoglobin and phenol red were determined as in Fig. 2A. Arrows indicate the fraction which contained the maximum concentration of the specified substance.

which migrates as Peak 2 does so because it is bound to some milk protein, livers from lactating mice were homogenized and NAL synthetase was partially purified using the same procedures as for the isolation of this enzyme from the mammary gland. In Fig. 3 it is apparent that liver NAL synthetase migrates predominately as a single peak. Arginase and ornithine transcarbamylase were also assayed in this preparation and their activities peak at a molecular weight greater than NAL synthetase. Rat arginase has a molecular weight of 140 000 and ornithine transcarbamylase has a molecular weight of 156 000 (see ref. 15). In the mouse liver, NAL synthetase apparently has a molecular weight between that of arginase and hemoglobin and a plot of elution volume *versus* log molecular weight (as in Fig. 2B) indicates that the molecular weight of liver NAL synthetase is also between 100 000 and 130 000. Thus, the molecular weight of this enzyme in the liver suggests that the mammary-gland enzyme with the same molecular weight is not due to the binding of the 29 000 molecular weight NAL synthetase to a milk protein.

Dilution of lactose synthetase

When crude mammary-gland homogenates are diluted, the activity of lactose synthetase is proportional to the extent of dilution (Fig. 4A). Thus, it was a surprise when partially purified preparations of lactose synthetase (made by combining A, Peak 2, purified as in previous section; with B, bovine α-lactalbumin, purified in a similar manner; see ref. 6) did not give linear dilution curves. Fig. 4A illustrates a typical dilution experiment with partially purified A and B. The decrease in lactose-

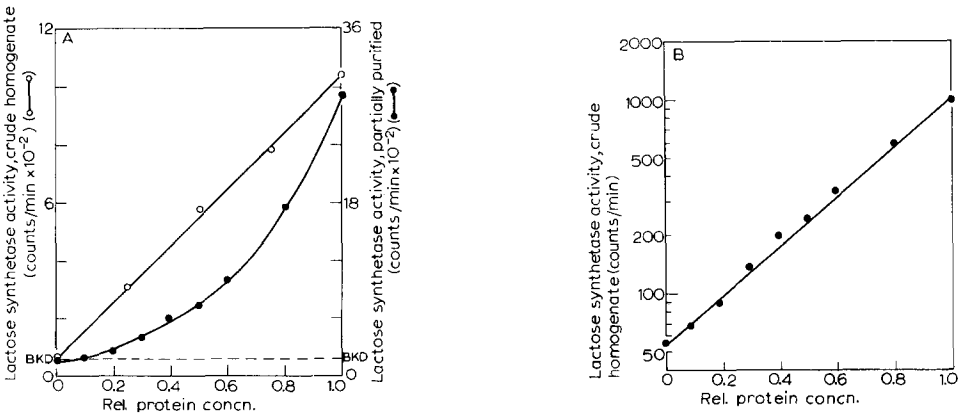

Fig. 4. A. Dilution of lactose synthetase. Open circles, dilution of a crude homogenate; closed circles, dilution of a partially purified preparation of A and B subunits. The crude homogenate was prepared from a lactating mouse (15 days *post partum*) as described in MATERIALS AND METHODS, including filtration through a Bio-Gel P-6 column. It was then diluted with Tris–Mg^{2+} buffer. The extent of dilution is illustrated on the abscissa as relative protein concentration. The partially purified preparation was made by combining mouse A subunit (NAL synthetase from Peak 2, see Fig. 2) with partially purified bovine α-lactalbumin (see ref. 6); both subunits were in Tris–Mg^{2+} buffer. The mixture was then diluted with Tris–Mg^{2+} buffer to the indicated relative protein concentrations. Bkd (background) is equivalent to counts/min obtained without the substrate, glucose, present in the reaction mixture. B. Semilog plot of dilution of partially purified preparation of lactose synthetase. The data in A has been re-plotted to illustrate the fact that the dilution of partially purified preparations of lactose synthetase results in an exponential loss of activity.

synthetase activity upon dilution of these preparations is exponential as illustrated in Fig. 4B.

Restoration of lactose-synthetase activity by recombining A and B subunits

The unexpected results of the dilution experiments led to a comparison of the recombination characteristics of unpurified with partially purified preparations of the A and B subunits.

The subunits of bovine lactose synthetase can be partially separated by ultracentrifugation after EDTA treatment since the A subunit is bound to the endoplasmic reticulum and the B subunit becomes dissociated from A[16]. The same separation technique works for mouse lactose synthetase. The mammary glands of a lactating mouse (20 days *post partum*) were homogenized in 20 mM Tris–HCl, 8 mM EDTA, and 20% glycerol (pH 7.5); 2 ml of this homogenate were layered over 5 ml of the same buffer in 25% glycerol, and overlaid by 6 ml of the same buffer without glycerol. The step-gradient thus formed was centrifuged for 3 h at 283 000 × *g* using an International centrifuge and rotor SB 283. (Under these conditions 95% of the microsomal enzyme, $NADH_2$-cytochrome *c* reductase (EC 1.6.2.1), is removed from the middle layer into the bottom layer and the pellet.) The upper layer (containing fatty material) was discarded, the original middle layer (now called the supernatant), and the pellet (microsomes) were saved. The pellet was resuspended by gentle homogenization in 20 mM Tris–HCl, 8 mM EDTA, and 20% glycerol (pH 7.5). Both microsomal and supernatant fractions were then eluted separately through a 1.5 cm × 25 cm column of Bio-Gel P6 using a 20 mM Tris–HCl, 20% glycerol buffer (pH 7.5). Gel filtration through P6 was necessary in order to remove EDTA since it completely inhibits lactose synthetase.

The microsomes always exhibited some lactose-synthetase activity, indicating that complete removal of the B subunit from the microsomal-bound A subunit was

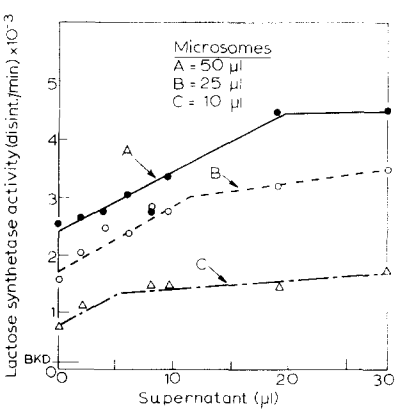

Fig. 5. Restoration of lactose-synthetase activity by recombination of three different concentrations of microsomes with supernatant fraction. Microsomes and supernatant fractions were prepared as described in text. Lactose-synthetase assays were conducted for 30 min at 40°. The initial concentration of glucose was 20 mM; $MnCl_2$, 13 mM; UTP, 5 mM; Tris–HCl (pH 7.5), 13 mM; glycerol, 13%; UDP-[$^{14}C_6$]galactose 3.3 μM (24 000 disint./min). The final reaction volume was always 150 μl. The radioactive products were isolated as described in MATERIALS AND METHODS. Under these conditions the supernatant fraction alone had no lactose-synthetase activity. Bkd has same meaning as in Fig. 4.

not achieved. However, the supernatant fraction had no lactose-synthetase activity under the conditions of these assays (see legend, Fig. 5) suggesting that only the B subunit was present in this fraction. Fig. 5 illustrates the recombination of three different quantities of microsomes with supernatant fraction. Note that the slopes of the saturation curves are approximately the same regardless of the concentration of microsomes used.

When the same type of experiment as is illustrated in Fig. 5 was performed using partially purified A and B subunits the results were quite different. Fig. 6 illustrates the recombination of five different concentrations of the A subunit (meas-

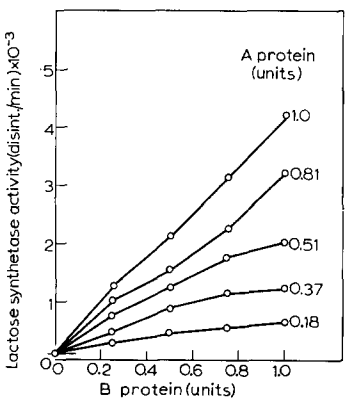

Fig. 6. Recombination of partially purified A and B subunits of lactose synthetase. The A subunit was taken from Fraction 33 (Fig. 2) and the B subunit was taken from Fraction 48 (Fig. 2). Lactose-synthetase assays were conducted for 20 min at 37° under standard conditions (see MATERIALS AND METHODS). Bkd, same as in Fig. 4. The units of both A and B subunits are arbitrary; however, one unit of A transfers 3675 disint./min galactose from labelled UDPGal to *N*-acetylglucosamine in 20 min at 37° under standard conditions.

ured with NAL-synthetase assay) with various concentrations of the B subunit. In this experiment the B subunit did not saturate the A subunit regardless of the quantity of A used. The slopes of the saturation curves are proportional to the amount of A subunit used rather than being constant as in Fig. 5.

DISCUSSION

Lactose-synthetase preparations from the mouse were found to have properties which closely resembled preparations of this enzyme isolated from the cow and rat. The B subunit (α-lactalbumin) of this two-subunit enzyme has previously been shown to have a molecular weight of approx. 15 000 daltons. The amino acid sequence of α-lactalbumin is similar to that of egg white lysozyme[17]. During the course of these experiments, the molecular weight of the A subunit of lactose synthetase was determined for the first time. Gel filtration of $(NH_4)_2SO_4$-fractioned mouse mammary gland homogenthrates ough Sephadex G-100 columns revealed three peaks of NAL synthetase (the A subunit of lactose synthetase) activity. The minimum molecular. weight was shown to be 29 000 daltons. However, the majority of mouse A subunit

eluted with a molecular weight near 110 000. The A subunit has been found in several tissues, whereas the B subunit is probably restricted to the mammary gland. Liver NAL synthetase elutes from a Sephadex G-100 column as a single peak with proteins having a molecular weight near 110 000. It is unknown at the present time whether the higher molecular weight A subunit is a multimer of the 29 000 dalton material or an artifact due to binding of the 29 000 molecular weight material to some other protein.

When unpurified preparations of lactose synthetase are diluted, the resulting activity is proportional to the extent of dilution. The reconstitution of lactose-synthetase activity by combining microsomes and supernatant fractions proceeds in a manner which suggests that once reassociated, the subunits do not readily dissociate (Fig. 5). However, when the two subunits of lactose synthetase were partially purified and then recombined, dilution resulted in an exponential reduction of activity (Fig. 4B). Experiments involving the reconstitution of lactose synthetase from purified A and B subunits indicate that the subunits are in a state of chemical equilibrium (Fig. 6).

These differences between crude homogenates and partially purified preparations are best explained by a model in which the A an B subunits form an active complex, AB, *via* Reaction 1, as was proposed by BRODBECK *et al.*[1]. The complex freely dissociates back to A and B.

$$A + B \rightleftharpoons (AB) \tag{1}$$

However, *in vivo* and in unpurified preparations the AB complex exists in combination with a third, as yet unspecified component, Z, as an ABZ complex. The most readily observed property of ABZ is that in the presence of Z, the A and B subunits do not dissociate as rapidly as the AB complex alone.

Thus, the explanation of the recombination and dilution experiments is based on the laws of mass action and chemical equilibrium. In the dilution experiments with partially purified A and B, the concentration of the active AB complex is dependent upon the rate of formation of the AB complex and the rate of dissociation; therefore, it can be treated as a general case of chemical equilibrium in which the concentration of the products divided by the concentration of the reactants is a constant. In Eqn. 1 the equilibrium constant, K, can be expressed as:

$$K = \frac{[AB]^x}{[A]^m [B]^n} \tag{2}$$

where A and B are reactants, AB is the product, and n, m, and x are equal to the number of molecules combining in a balanced equation. So, what we are determining when partially purified A and B are combined is a K which is directly related to the lactose-synthetase activity since neither A nor B alone have lactose-synthetase activity. Thus it is apparent that if an enzyme solution that is composed of two subunits which dissociate is diluted in half, the resultant enzyme activity will be $\frac{1}{4}$ of the original instead of $\frac{1}{2}$. (*e.g.* if $K = 1$ and the concentration of A and B equal 1, then $1 = [AB]/[1][1]$ or $AB = 1$; then after a 1:1 dilution of the enzyme solution $1 = [AB]/[\frac{1}{2}][\frac{1}{2}]$ or $AB = \frac{1}{4}$.)

In the recombination experiments (Fig. 6) with partially purified A and B, the equilibrium depends upon the concentration of both A and B, which explains why

the slopes of the recombination curves using varying amounts of A are different; however, the individual recombination curves are linear since the concentration of only one of the subunits is being changed at a time.

The properties of lactose synthetase in experiments in which unpurified preparations of the enzyme were used are inconsistent with the properties, discussed above, of partially purified preparations; therefore a third component, Z, has been proposed. If instead of having a readily dissociable AB complex one has an ABZ complex that does not easily dissociate then the dilution of the ABZ complex will result in enzyme activity that is proportional to the extent of dilution as in Fig. 4A. Also, recombinations of B with varying amounts of A, when Z is present to prevent dissociation of the AB complex that is formed, will proceed along a single slope until the A subunit becomes saturated (see Fig. 5).

Some of the decay phenomena of lactose synthetase can be explained using this model. For instance the differential loss of AB activity compared to A may be due to a gradual dissociation of the AB complex from Z. Free from Z, the AB complex would then freely dissociate into A + B, since the equilibrium constant for the AB complex is apparently a different order of magnitude from that of the ABZ complex, *i.e.*, the activity of lactose synthetase could decline even though the concentration of the A and B subunits might remain the same. The dissociation of AB from Z in crude homogenates is probably occuring in a system that is also under the influence of general enzymatic degradation (Fig. 1). Likewise, in Table I sonication may be destroying Z and thus subjecting the AB complex to a more rapid dissociation, rather than destroying B which is the apparent explanation. It is particularly unconvincing to think of sonication destroying B since (1) B has a molecular weight of about 15 000 and small proteins are generally more stable than large ones, (2) B can be boiled without loss of activity and (3) B can be acid precipitated and redissolved without loss of activity.

The nature of the Z component in this system is unknown, although it is conceivable that it is a membrane protein, especially since the A subunit of lactose synthetase is known to be bound to the endoplasmic reticulum[16]. The role of membrane, or structural, proteins in the regulation of enzymatic activity has only begun to be investigated. As an example of the importance of structural proteins, WOODWARD AND MUNKRES[18] have demonstrated that the activity of many mitochondrial enzymes, including the well analysed enzyme, malate dehydrogenase, are altered when mutations in the structural proteins to which these enzymes are normally bound are produced. Regardless of the nature of Z, the extent of binding of the AB complex to the Z component adds a new dimension to the potential modulation of lactose-synthetase activity.

Should the Z component be a membrane protein, the subunits of lactose synthetase should prove to be useful in an assay for membrane protein, since in the presence of Z the total activity of a mixture of lactose synthetase A and B subunits should increase due to a shift in the equilibrium in Eqn. 1 to the right.

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