Characteristics of Arginases from Ureotelic and Non-Ureotelic Animals

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1. Livers of ureotelic and uricotelic animals possess the capacity, to a varying extent, of hydrolysing L-arginine and other guanidino compounds. 2. Three different enzymes responsible for such activity have been separated and partially characterized. They are the 'ureotelic' arginase, the 'uricotelic' arginase (both of them specific for L-arginine) and the guanidinobutyrate ureohydrolase (able to hydrolyse β -guanidinopropionate, γ -guanidinobutyrate and D-arginine but not L-arginine or guanidinoacetate). 3. The implication of the advent of a 'ureotelic' arginase in the integration of the Krebs-Henseleit cycle is discussed.

For many years it was thought that arginase was absent from the liver of uricotelic animals. This fact has been commonly stated as the rule of Clementi (1914, 1919). However, recent work demonstrated a low arginase activity in the liver of chicken (Tamir & Ratner, 1963) and other uricotelic species (Mora, Martuscelli, Ortiz-Pineda & Soberón, 1965). This arginase differs from that present in the liver of ureotelic animals in having a higher K_m for L-arginine (100–200 mM and 10–20 mM respectively; Mora *et al.* 1965).

This work establishes that the arginase that participates in ureotelism is a different protein from the arginase present in the liver of uricotelic animals. Evidence is also presented for the existence of another enzyme capable of hydrolysing guanidino compounds (guanidinobutyrate ureohydrolase).

MATERIALS AND METHODS

Animals. The animals used were obtained from commercial and private sources. For the study of the pattern of development of the enzymes, fertile eggs from white Leghorn hens were obtained from a commercial hatchery. Embryos were harvested at the indicated times and the livers and kidneys were removed and pooled in chilled beakers.

Chemicals. D-Arginine and serum albumin were purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Other chemicals were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.

Preparation of tissues and enzyme assays. All tissues studied were homogenized in cold water (4°) with the aid of a Potter-Elvehjem device. Arginase was measured as described by Brown & Cohen (1959). The incubation mixture for the formation of urea from γ -guanidinobutyrate and β -guanidinopropionate was similar to that for the arginase assay, since the same conditions were found to be optimum for both assays (Fig. 1).

Purification of enzymes. Arginase from rat liver was purified according to the method of Schimke, Brown & Smallman (1963). The preparation obtained represented about 1000-fold purification, and gave a single band when run in polyacrylamide-gel electrophoresis at pH 8.1.

The arginase from the liver of uricotelic animals was purified from homogenates made with 25 g. of each tissue/100 ml. of a solution containing Triton X-100 (0.5%), $MnCl_2$ (0.5 mM), glycine-NaOH buffer, pH 9.5 (0.25 M) and KCl (0.1 M). All manipulations were carried out at 4°. Step 1: the homogenate was centrifuged at 17000g for 20 min. and the precipitate discarded. Step 2: the supernatant was heated at 60° for 10 min. and the denatured protein separated by centrifugation. Step 3: the supernatant was added to an equal volume of acetone at -10°

Fig. 1. Assay conditions for guanidinobutyrate ureohydrolase. The ordinate for the progress curve (a) is given as μ moles of urea produced by 0.1 ml. of a 10% chicken-liver homogenate; the ordinates for the enzyme concentration against activity, curve (b), and for the pH against activity, curve (c), are μ moles of urea produced/hr./0.1 ml. of homogenate. The incubation system contained 50 μ moles of L-arginine, 25 μ moles of $MnCl_2$ and 25 μ moles of glycine buffer (●) or tris buffer (○).

with stirring, the precipitate collected by centrifugation at 15000g for 5 min. and the supernatant discarded. Step 4: the precipitate was resuspended in 30 ml. of 0.1M-tris-HCl buffer, pH 7.5, containing MnCl_2 (0.05M), and the suspension was centrifuged at 15000g for 15 min. Step 5: the precipitate was resuspended in 10 ml. of the tris- MnCl_2 solution and chromatographed on a DEAE-cellulose column (15mm. $\times$ 350mm.) previously equilibrated with 0.01M-tris-HCl buffer, pH 7.0. The enzyme passed through the column with the exclusion volume. Step 6: to the supernatant from step 4 a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ was gradually added and the precipitates from 0-35%, 35-50% and 50-60% saturation were collected by centrifugation. Each precipitate was resuspended in 5 ml. of the tris- MnCl_2 solution and dialysed against the same solution before the assay of the enzyme activity. Step 7: 12 ml. of the resuspended and dialysed 50-60% saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate from lizard liver and the 35-50% saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate from chicken liver and kidney were chromatographed on DEAE-cellulose. The elution started with 25 ml. of 0.1M-tris-HCl buffer, pH 8.0; it was followed by sequential elution with fractions of the same buffer containing 0.05M-L-arginine (25 ml.), 0.2M-L-arginine (25 ml.) and 0.5M-L-arginine (50 ml.).

Protein was determined by the procedure described by Lowry, Rosebrough, Farr & Randall (1951).

Immunochemical studies. Similarities between the antigenic properties of the enzymes studied were also explored. A rabbit antibody to a purified homogeneous preparation of rat-liver arginase was kindly supplied by Dr Robert T. Schimke, National Institutes of Health, Bethesda, Md., U.S.A. The antigen-antibody reaction was assayed by the Ouchterlony (1953) double-diffusion technique and also by determining the enzyme activity that remained in the supernatant after carrying out the antigen-antibody reaction either at 4° during 24-48 hr. or at 35° during 30 min.

Molecular-weight determinations. These were carried out by means of a sucrose-gradient analysis according to the method of Martin & Ames (1961). Serum albumin, recrystallized five times, was used as standard. Because it was found that sucrose interferes with the colorimetric determination of urea, arginase was assayed by the appearance of ornithine (Chinard, 1952), and the hydrolysing activity towards γ -guanidinobutyrate was measured by the disappearance of guanidino groups (Van Pilsum, Martin, Kito & Hess, 1956).

RESULTS

Guanidino ureohydrolases in the livers of ureotelic and uricotelic animals. Because the arginases studied differ in their K_m for L-arginine it was considered that they could vary in the mechanism responsible of the hydrolysis of this amino acid. The specificity of the liver arginase from ureotelic animals was worked out by Thomas (1913), Thomas, Kapfhammer & Flaschentrager (1922), Grassman, Hörman & Janowsky (1958) and Greenberg (1960). There are specific requirements involving the α -carbon atom and the guanidino group, in addition to a requirement for optimum chain length. It was assumed therefore that the mechanism of

enzyme action could be elicited by the simultaneous presence of two substrate molecules, one contributing the functional groups required at one end of the L-arginine molecule and the second one providing those necessary at the other end. Thus we investigated the behaviour of liver homogenates of ureotelic and uricotelic animals with combinations of guanidine, methylguanidine, guanidinoacetate, β -guanidinopropionate or γ -guanidinobutyrate and straight-chain L- α -amino acids of different chain length. Although rat-liver arginase did not produce urea from any of these combinations, chicken liver hydrolysed γ -guanidinobutyrate and β -guanidinopropionate even in the absence of L- α -amino acids.

A general survey of the ability of liver homogenates from ureotelic and uricotelic animals to hydrolyse a number of guanidino compounds is shown in Table 1. For comparison, the hydrolysing activities towards L-arginine and the other guanidino compounds were measured at 0.1M-substrate concentration. No clear relationship exists between the hydrolysis of L-arginine and the other guanidino compounds. However, the ratio of the hydrolytic activities towards γ -guanidinobutyrate and β -guanidinopropionate was fairly constant for almost all the livers assayed. These results seem to indicate that the arginase present in ureotelic species bears no relationship to the hydrolysing capacity towards the other guanidino compounds, but that it is not certain that such a relationship does not occur in the uricotelic animals.

Distinction between arginase and γ -guanidinobutyrate-ureohydrolase activities. To find out if a single enzyme could hydrolyse L-arginine and γ -guanidinobutyrate another approach was made.

Table 1. *Hydrolysis of guanidino compounds by livers of different animal species*

Results are expressed as μ moles of urea produced/hr./g. of wet tissue. Determinations were run at 0.1M-substrate concentration. B.L.D., Below limits of detection.

Species	L-Arginine	β -Guanidino-propionate	γ -Guanidino-butyrate
Rat	29000	B.L.D.	B.L.D.
Mice	42750	B.L.D.	B.L.D.
Frog	57000	8.0	48.0
Axolotl (non-metamorphosed)	27280	135.0	481.0
Turtle (<i>Pseudemys</i>)	38400	100.0	300
Turtle (<i>Kinosternon</i>)	30200	33.0	100
Turtle (<i>Gopherus</i>)	23400	—	360
Lizard	1800	120	900
Chicken	90	90	455
Rattlesnake	102	119	304

A characteristic pattern of development has been described for different enzymes which reflects the specific regulatory control system or systems that operate on them. If the hydrolysis of two substrates is attributable to a single protein the ratio of their respective activities must remain constant throughout the indicated process. But, if two different proteins are responsible for the catalytic conversion of the two substrates, their activities during ontogenesis should differ, unless they are under the same regulatory control mechanism. Alternatively, the coincidental appearance of the two patterns is a rather remote possibility. Fig. 2 shows the pattern of development in chicken liver and kidney of the ureohydrolases studied. Chicken kidney was also included because it has the peculiarity of moderately high arginase activity with a K_m corresponding to that found in livers of ureotelic animals and can also hydrolyse γ -guanidinobutyrate. The ratio of the hydrolytic activities toward L-arginine and γ -guanidinobutyrate changes during ontogenesis, a fact that is more marked in kidney. Also the K_m values for each activity remain constant; the K_m for the hydrolytic activities towards γ -guanidinobutyrate is the same in liver and kidney, although the development pattern

varies from one organ to the other. Kidney arginase is inhibited by substrate, a fact consistently found with arginase from livers of ureotelic animals; this is illustrated in Fig. 3, which shows that the

Fig. 3. Kinetic characteristics of liver arginases from different animal species. (a)–(f) Reciprocal plots ($10^3/\text{velocity}$ against $1/M$) of the activities of arginases expressed as $\mu\text{moles of urea produced/hr.}$ The amount of tissue was 2 mg. for rat (a), 2 mg. for semi-aquatic turtle (b), 50 mg. for snake (c), 2 mg. for mouse (d), 2 mg. for human (e) and 40 mg. for lizard (f). Other conditions were as described in Fig. 1.

Fig. 2. Development and kinetic characteristics of guanidino ureohydrolases in the chicken. The change in the capacity to produce urea from arginine (○) and guanidinobutyrate (●) in kidney (a) and liver (b) is illustrated. The activities were measured at 0.1M-substrate concentration. (c)–(f) Reciprocal plots ($1/\text{velocity}$ against $1/M$) of the activities of arginase (○) and guanidinobutyrate ureohydrolase (●) at different stages of development of the respective activities in the kidney (c and e) and the liver (d and f). The numbers indicate the days before or after hatching when the study was carried out. Incubation systems contained either 30 mg. (liver) or 15 mg. (kidney) of wet tissue in a final volume of 1.0 ml. at pH 9.5. The substrate concentration was varied as indicated.

arginases from human, semi-aquatic turtle, rat and mouse livers have this peculiarity, whereas those from snake and lizard livers are not inhibited by an excess of arginine. The K_m of the arginase present in the ureotelic species is about one-tenth of that in the uricotelic animals as reported by Mora *et al.* (1965).

Another criterion used to differentiate enzymes is based on their physical separation by the methods of protein fractionation. The chicken and lizard livers were suitable tissues for this purpose, since the ratio of hydrolytic activities towards L-arginine and γ -guanidinobutyrate was different for every organ (Table 1); chicken kidney was also studied. Table 2 shows the specific activities with L-arginine and γ -guanidinobutyrate as substrates obtained through the different steps of the purification procedure, and Fig. 4 illustrates a characteristic elution pattern of the two activities. Although the ratio between both activities was kept constant through several purification steps it substantially changes at a point where it was possible to free the arginase from γ -guanidinobutyrate ureohydrolase. Further, a chicken liver with very low arginase content was submitted to the same purification scheme as before and a preparation free from arginase and able to hydrolyse γ -guanidinobutyrate was obtained, which represented a 45-fold purification of the latter enzyme (see Table 3).

The activity of the guanidino ureohydrolases under study towards several guanidino compounds

is presented in Table 3. From these data and also from the findings presented above some characteristics of the two different proteins responsible for the production of urea from the substrates tested can be inferred. One of the proteins hydrolyses only

Fig. 4. Chromatographic separation of guanidino ureohydrolases of chicken liver on DEAE-cellulose. The arginase (○) and the guanidinobutyrate ureohydrolase (●) were assayed under the conditions indicated for Fig. 1, with glycine as buffer. For the conditions of chromatography see the Materials and Methods section.

Table 2. Separation of L-arginine ureohydrolase and γ -guanidinobutyrate ureohydrolase in tissues of two animal species

Activities are expressed as μ moles of urea formed/hr./mg. of protein.

Purification step	Lizard liver			Chicken liver			Chicken kidney		
	(A) L-Argi- nine	(B) γ -Guanidino- butyrate	A/B	(A) L-Argi- nine	(B) γ -Guanidino- butyrate	A/B	(A) L-Argi- nine	(B) γ -Guanidino- butyrate	A/B
Homogenate	15.8	5.7	2.88	0.7	2.4	0.29	37.2	1.41	26.6
(1) 17000g supernatant	17.1	6.6	2.58	0.5	2.0	0.25	47.7	1.91	46.2
(2) Supernatant after heat denaturation	51.0	20.5	2.49	6.0	21.4	0.28	54.6	1.38	39.6
(3) Soluble acetone precipitate	39.0	35.4	1.10	4.0	13.7	0.29	64.6	4.1	15.65
(6) 35–50% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate	—	—	—	11.2	74.5	0.45*	8.1	12.0	0.67*
(6) 50–60% satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate	58.0	71.0	0.82*	10.0	50.0	0.20	—	—	—
(7) DEAE-cellulose chromatography	173.0	240.0	0.72	11.0	230.0	0.05	28.0	120.0	0.2
(4) Non-soluble acetone precipitate	67.0	2.4	27.90	1.5	4.41	0.34	161.0	3.7	43.17
(5) DEAE-cellulose chromatography of (4)	164.0	0	—	—	—	—	420	0	—

* Fraction submitted to chromatography.

Table 3. *Guanidino-ureohydrolase activities toward different guanidino compounds*Values are expressed as μ moles of urea formed/hr./mg. of protein.

Enzyme source	L-Arginine	D-Arginine	β -Guanidino-propionate	γ -Guanidino-butyrate
Lizard liver (step 5)	164.0	0	0	0
Ox liver*	305.0	0	0	0
Chicken liver (step 6)	10.0	36.4	16.6	50.0
Chicken liver†	0	1.8	0.9	2.8

* The ox-liver arginase was purified 45-fold by Dr Mario Castañeda.

† This fraction is not included in Table 1; it corresponds to the 45-fold purification referred to in the text.

Table 4. *Effect of Mn^{2+} ions on the capacity of chicken liver to hydrolyse guanidino compounds*

Results are expressed as μ moles of urea produced/hr./g. of wet tissue. The complete system contains 100 μ moles of substrate, 25 μ moles of glycine-NaOH buffer, pH 9.5, and liver homogenate (25 mg. of wet tissue) in a final volume of 1.0 ml.; 25 μ moles of $MnCl_2$ were added where indicated.

Substrate	With Mn^{2+}	Without Mn^{2+}	Ratio
L-Arginine	170	70	2.43
D-Arginine	436	182	2.35
β -Guanidinopropionate	345	152	2.27
γ -Guanidinobutyrate	520	240	2.17

L-arginine, whereas the other splits D-arginine, β -guanidinopropionate and γ -guanidinobutyrate but not L-arginine. Thus the arginases present in the livers of uricotelic and ureotelic animals have a similar substrate specificity. Neither of these enzymes produces urea from guanidinoacetate. The guanidinobutyrate ureohydrolase splits D-arginine and two guanidino acids in the same proportions (2:1:3). Therefore we may conclude that the chicken-liver preparation from step 6 still contains both enzymes.

There are similarities between arginase and guanidinobutyrate ureohydrolase, since both activities are enhanced by Mn^{2+} ions (Table 4) and both have their pH optimum at about 9.5 (Fig. 1).

Distinction between 'ureotelic' and 'uricotelic' arginases. The antigenic peculiarities of the guanidino ureohydrolases present in different ureotelic and uricotelic species were investigated. By means of the Ouchterlony (1953) double-diffusion technique a precipitation band was clearly observed when the supernatant from rat-liver homogenate and the purified rat-liver and ox-liver arginases were allowed to diffuse against rabbit antibodies to a purified homogeneous rat-liver arginase. The same bands did not appear with the arginases purified from chicken and lizard liver, nor with the purified preparation of guanidino-

Fig. 5. Effect of rabbit antibodies against a purified rat-liver arginase on the activity of the guanidino ureohydrolases from ureotelic and uricotelic animals. The enzyme activity is expressed on a percentage basis: 100% is taken as 34 units of lizard-liver arginase ($\circ$), 42 units of lizard-liver guanidinobutyrate ureohydrolase (Δ) and 150 units of rat-liver arginase ($\square$). The unit is the amount of enzyme that converts 1 μ mole of substrate/hr. The specific activities (units/mg. of protein) of the preparations used were 65, 75 and 5000 respectively. The incubation mixture (0.5 ml. final volume) was allowed to react for 30 min. at 35°.

butyrate ureohydrolase from lizard liver. In agreement with these findings we observed that rabbit antibodies decreased by approx. 95% the enzymic activity of the purified rat-liver arginase and produced no effect with those preparations that failed to react immunologically with the same antibodies (Fig. 5). The results were similar when the mixtures were allowed to react for 48 hr. at 4° or 30 min. at 35°. Further, when the purified arginase from lizard liver was incubated with the rabbit antibodies and the mixture allowed to react afterwards with the rat arginase, a precipitate appeared that was accompanied by a decrease in

Fig. 6. Determination of molecular weight by sucrose-gradient analysis of purified guanidino ureohydrolases from ureotelic and uricotelic animals. A, Serum albumin; B, purified arginase from rat liver; C, partially purified guanidinobutyrate ureohydrolase from lizard liver; D, partially purified arginase from lizard liver. Conditions of the assay: the mixture of proteins in the sucrose gradient was centrifuged at 100000g for 16hr. Enzyme activity is expressed as μ moles of substrate converted into product/hr./0.094 ml. of effluent. The guanidinobutyrate-ureohydrolase activities have been divided by 2 to fit them on to the indicated scale (see the Materials and Methods section). The molecular weights of the two arginases and the guanidinobutyrate ureohydrolase were determined in different runs.

the enzyme activity corresponding to the rat arginase, similar to what is observed in the absence of the arginase from uricotelic species.

Of great significance is that a distinct band was obtained when the supernatant from a uricotelic terrestrial-turtle liver homogenate was incubated with the same antibodies. This species has a high arginase activity with a K_m of the same order of magnitude as that observed for the arginase from livers of ureotelic animals (Mora *et al.* 1965). Fig. 6 shows the distribution in a sucrose-gradient analysis of the purified proteins being studied. The values obtained for 'ureotelic' arginase (rat liver), 'uricotelic' arginase (lizard liver) and guanidinobutyrate ureohydrolase (lizard liver) were 138 000, 276 000 and 252 000 respectively. The molecular weight of 138 000 corresponding to the 'ureotelic' arginase (rat liver) agrees well with that reported by Schimke (1962). The preparations of the 'uricotelic' arginase and guanidinobutyrate ureohydrolase used for the molecular-weight determinations were respectively 40- and 100-fold purified. The specific activity of the enzymes from fractions taken at their corresponding maximum

peaks was further increased fivefold. A purified homogeneous preparation of rat-liver arginase was used for the study (see the Materials and Methods section).

DISCUSSION

The results establish that the arginase present in the liver of ureotelic animals is a different protein from that in the liver of uricotelic species. They differ in several respects: (a) K_m ; (b) inhibition by an excess of substrate; (c) antigenicity; (d) molecular weight.

The difference in K_m values is significant because it is maintained through purification and ontogenesis. There is a close relation between K_m values and antigenic properties. All the arginases present in the liver of the ureotelic animals tested (man, dog, monkey, cow, cat, pig, ox) cross-react with the rabbit antibodies prepared against purified rat-liver arginase (R. T. Schimke, personal communication), and they have K_m values 10–20 mM (Bach & Killip, 1961; Mora *et al.* 1965). The arginases present in the liver of the uricotelic animals studied (lizard and chicken) fail to cross-react with the same antibodies and they have K_m values 100–200 mM.

However, both arginases are highly specific for L-arginine, their activity is enhanced by Mn^{2+} ions and the optimum pH for their action is about the same. The hydrolysis of L-arginine then can be accomplished by two proteins with a high degree of specificity. These proteins have a different structure (as inferred from their antigenic properties), which should be a consequence of their different compositions. They are present in animals with different nitrogen-excretion patterns, and therefore they should be engaged in different metabolic functions. The 'ureotelic' arginase is able to hydrolyse endogenous L-arginine with great efficiency. The 'uricotelic' arginase is present in the livers of animals that do not possess the enzymes of arginine biosynthesis (Tamir & Ratner, 1963; Mora *et al.* 1965), and its specific role in intermediary metabolism cannot be ascertained; however, that this arginase might function in a different manner from that in ureotelism is indicated by the fact that *Neurospora crassa* can hydrolyse only exogenous arginine and cannot hydrolyse endogenous arginine (J. Mora, R. Tarrab, M. Castañeda & L. F. Bojalil, unpublished work).

It is probably that 'ureotelic' arginase should have specific properties to fulfil its function in the operation of the urea cycle, i.e. in addition to a proper catalytic site, possibly a suitable structure must be also required; this is a general problem relative to isoenzymes, enzyme ecology and/or

compartmentalization. The establishment of ureotelism is the result of integration, during evolution, of some of the enzymes that participate in the arginine biosynthesis in unicellular organisms with at least two distinct proteins: (a) a carbamoyl phosphate synthetase, different from carbamate kinase, having a low K_m for ammonia and requiring 2 mol. prop. of ATP (Cohen & Brown, 1960); (b) a 'ureotelic' arginase with the characteristics reported in the present paper.

If 'ureotelic' arginase can be related to the operation of ureotelism two other observations are of importance. First, that the arginase activity present in the liver of the uricotelic terrestrial turtle represents a remnant from its ureotelic past, as we have previously suggested (Mora *et al.* 1965); secondly, since the arginase of chicken kidney seems to have the peculiarities of the enzyme from ureotelic animals, it cannot be considered fortuitous that ornithine transcarbamoylase, argininosuccinate synthetase and argininosuccinate lyase, other urea-cycle enzymes, are present in the same organ (Tamir & Ratner, 1963). Incidentally, the low activity of the same enzymes detected in the lizard liver accompanied by an arginase activity ten times that usually found in other uricotelic species, where the other enzymes are completely absent (Mora *et al.* 1965), suggests that it might contain some 'ureotelic' arginase. The experimental procedure employed does not rule out this possibility since purified preparations were used for the antigenic studies. Conversely, it is possible that ureotelic animals have also 'uricotelic' arginase.

The finding of a third distinct protein able to hydrolyse guanidino compounds, the guanidinobutyrate ureohydrolase, which utilizes as substrate the compounds β -guanidinopropionate, γ -guanidinobutyrate and D-arginine, but not L-arginine and guanidinoacetate, is also relevant. To our knowledge such enzymic activity has not been previously reported in the liver and kidneys of ureotelic and uricotelic animals.

The possible metabolic role of this enzyme is a matter of speculation; however, the presence of γ -guanidinobutyrate has been demonstrated in animal tissues (Pisano, Abraham & Udenfriend, 1963). We did other experiments showing that an uncharacterized *Streptomyces* sp. isolated in our Laboratory can grow on guanidinobutyrate as the sole source of nitrogen with the concomitant

appearance of urea in the incubation medium; it can also grow on γ -aminobutyrate, and cell-free extracts from this organism produced urea from guanidinobutyrate but not from arginine. However, cell-free extracts from *Neurospora crassa* 74A capable of growing on arginine as the only nitrogen source have arginase activity but not guanidinobutyrate-ureohydrolase activity (J. Mora, J. Martuscelli, I. Magaña & L. F. Bojalil, unpublished work).

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