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Maleic Acid-Induced Inhibition of Amino Acid Transport in Rat Kidney

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Maleic acid, the *cis* isomer of fumaric acid, has long been known to be a potent inhibitor of several enzyme systems *in vitro* (Morgan & Friedmann, 1938*b*), but its effects *in vivo* have been investigated only recently. Berliner, Kennedy & Hilton (1950) reported that acidotic dogs, given maleic acid intravenously, failed to acidify their urine. These authors also noted a consistent increase in urinary sodium, phosphorus and bicarbonate as well as a decrease in the maximal concentration of glucose able to be reabsorbed by the kidneys (T_m glucose) in these poisoned animals. Harrison & Harrison

(1954) found that the administration of maleic acid to rachitic rats resulted in aminoaciduria, glycosuria and phosphaturia, and commented on the similarity of these findings to those in human subjects exhibiting 'the Fanconi syndrome'. Angielski, Niemiro, Makarewicz & Rogulski (1958), Angielski & Rogulski (1959) and Angielski, Rogulski & Madonska (1960) corroborated these findings in rats, documented the time-course of maleate poisoning and noted its effect on free thiol groups in kidney parenchyma. These workers also compared the effects of the administration of

maleate with alterations in renal function produced by other toxic substances such as mercury, arsenic, iodoacetate and phlorrhizin.

Although these results have clearly shown that maleic acid impairs renal transport mechanisms, the nature and site of this effect remain obscure. The present studies, undertaken to clarify the mode of action of this compound, and to correlate the observations *in vivo* with studies of transport in kidney, gut and muscle *in vitro*, indicate that maleic acid impairs the uptake of amino acids by kidney-cortex slices by retarding influx and accelerating efflux of substrate from slice cells. Further, our results suggest that this inhibitory effect is specific for kidney tissue and may be related to impaired intracellular metabolism rather than to a specific membrane effect.

EXPERIMENTAL

Studies in vivo. Eight male Sprague-Dawley rats, weighing 130–140 g., were housed in stainless-steel metabolism cages designed to separate urine and faeces, and were fed on ground Purina Chow and water *ad libitum*. After a 48 hr. acclimatization period, two successive control 24 hr. urine samples were collected. Each animal was then given a single subcutaneous injection of maleic acid (400 mg./kg.) dissolved in 0.9% NaCl solution and adjusted to pH 7 with NaOH. Urine was then collected continuously for 7 days, the collection period being 24 hr. with the exception of the first day after the administration of maleate when fractional urine samples were collected at 2, 12 and 24 hr. Urine was collected in graduated cylinders containing toluene as a preservative, and, after each collection period, the cage walls were washed with 20 ml. of distilled water and the washings combined with the voided urine. After measurement of the urine volumes, the samples were stored at -20° until analysed. Between collections each cage was thoroughly washed with distilled water and dried.

The following determinations were then performed on the urine samples; glucose according to the glucose-oxidase method (Saifer & Gerstenfeld, 1958); α -amino nitrogen by the colorimetric ninhydrin method of Rubinstein & Pryce (1959); quantitative individual free amino acid concentrations by using the column-chromatographic system of Moore, Spackman & Stein (1958).

Studies in vitro. Male Sprague-Dawley rats, weighing 140–180 g. were killed by stunning and decapitation. Slices of kidney cortex, liver and spleen were obtained with a Stadie-Riggs microtome. The techniques employed for the aerobic incubation of kidney-cortex slices in Krebs-Ringer bicarbonate buffer, pH 7.4, at 37° , and assessment of intracellular and extracellular radioactivity, were as described by Rosenberg, Blair & Segal (1961b). Total tissue water, determined by the difference between tissue weight after blotting and after drying in a crucible at 105° for 24 hr., was $80.2 \pm 1.0\%$ of the wet weight, and extracellular space, estimated with ^{14}C -labelled inulin, was $25.7 \pm 1.1\%$ of the wet weight of tissue (Rosenberg, Downing & Segal, 1962a; Robinson, 1950). Neither of these parameters was significantly altered by maleic acid (3 mM). Segments of

everted proximal jejunum, 0.3–0.5 cm. in length, were prepared according to the methods of Crane & Mandelstam (1960). Pooled everted jejunal segments from four or five rats were chilled in bicarbonate buffer at 4° before incubation. Three segments, weighing 40–80 mg., were placed in Erlenmeyer flasks containing 2.0 ml. of Krebs-Ringer bicarbonate buffer, pH 7.4, and the labelled substrate. The flasks were then gassed for 30 sec. with $\text{O}_2 + \text{CO}_2$ (95:5), sealed with rubber stoppers and incubated in a Dubnoff shaker for 30–60 min. After termination of the incubations, these tissues were handled in a fashion identical with that described for the kidney-cortex slices. Total tissue water and extracellular space estimations for jejunal segments were 84.3 ± 0.02 and $11.4 \pm 1.0\%$ respectively. The method of Kipnis & Cori (1957) was followed in the preparation of 'cut' diaphragm muscle. Amino acid uptake, in the presence and absence of maleic acid, was determined by methods identical with those described for the kidney-cortex slices. Total tissue water and extracellular space estimations with this tissue were 78.0 and 37.5% respectively, as reported by Kipnis & Cori (1957).

After incubation, samples of the aqueous tissue extracts obtained from kidney, gut or muscle were chromatographed on paper by using a single-dimension ascending system in butanol-acetic acid-water (4:1:2, by vol.). With the counting techniques described by Rosenberg *et al.* (1961b) more than 90% of the recovered radioactivity was uniformly found with the appropriate R_F for the specific amino acid studied. All radioactive samples were counted in an automatic liquid-scintillation spectrometer with an efficiency of 57%.

The $^{14}\text{CO}_2$ evolved during incubation studies with labelled maleic acid was collected as described by Rosenberg, Weinberg & Segal (1961a) and counted in an automatic liquid-scintillation spectrometer.

Materials. ^{14}C -labelled AIB* was obtained from Isotope Specialties Co. with a specific activity of 3.39 mc/m-mole. [^{14}C]Glycine (specific activity 1.19 mc/m-mole), L-[^{14}C]phenylalanine (specific activity 1.30 mc/m-mole), L-[^{14}C]lysine (specific activity 1.62 mc/m-mole), L-[^{14}C]histidine (specific activity 1.06 mc/m-mole) and [^{14}C]inulin (specific activity 1.0 mc/m-mole) were obtained from Volk Radiochemical Co. 1-Aminocyclopentane-1-[^{14}C]carboxylic acid (specific activity 2.62 mc/m-mole) was obtained from New England Nuclear Corp. Each of the above amino acids was shown to be chromatographically pure in a single-dimension ascending paper system with a butanol-pyridine-water solvent (1:1:1, by vol.). [^{14}C]Maleic acid anhydride (specific activity 1.20 mc/m-mole) and unlabelled 1-aminocyclopentane-1-carboxylic acid were purchased from the California Corp. for Biochemical Research. Unlabelled maleic acid, obtained from Coleman-Mathieson Inc., was dissolved in double-distilled water and neutralized to pH 7 with NaOH before addition to incubation flasks. An equivalent amount of Na^+ ion, as NaCl, was added to control flasks.

RESULTS

Effects of the administration of maleic acid in vivo. As shown in Fig. 1 a single subcutaneous injection of maleic acid (400 mg./kg.) was followed within

* Abbreviation: AIB, α -aminoisobutyric acid.

2 hr. by a marked diuresis and striking increases in the urinary excretion of glucose and α -amino nitrogen. These effects were maximal within the first 12 hr. and all toxic effects of the drug had

Fig. 1. Renal glycosuria and aminoaciduria produced by the administration of maleic acid (400 mg./kg.). Each point represents the mean value from eight animals. The vertical arrow (at 0 hr.) indicates the time of administration of maleate. —, Glucose; ----, α -amino nitrogen. Means $\pm$ S.E.M. were 115 ± 5 and 77 ± 3 μ g./hr. for glucose and α -amino nitrogen respectively before injection, and 4000 ± 120 and 413 ± 38 respectively in the 12-hr. period after injection.

disappeared within 72 hr. of administration, The rats appeared listless on the day of maleate poisoning, but perfectly normal thereafter. Blood samples, obtained 2 hr. after the administration of maleic acid, were analysed for glucose and α -amino nitrogen, and no differences from control animals were found. These findings, in agreement with those of Angielski *et al.* (1958), indicate that the glycosuria and aminoaciduria are renal in origin and not 'overflow' phenomena.

Quantitative determinations of individual urinary amino acids by ion-exchange chromatography were carried out on three rats during a control day, and during the first 12 hr. after the administration of maleate (Table 1). The excretion of taurine, serine, threonine, glutamic acid, glycine, alanine, valine, lysine and histidine were markedly increased after the injection of maleic acid, but little effect was noted on the other amino acids present in measurable quantities, These quantitative results support the impression of a generalized aminoaciduria obtained by paper chromatography.

Kidney tissue was examined histologically 1, 2 and 4 hr. after the administration of maleate. Routine haematoxylin-and-eosin staining revealed no abnormalities, and special stains did not show accumulation of glycogen or fat in the kidney parenchyma.

Effect of maleate on amino acid uptake by kidney-cortex slices in vitro. As shown in Table 2, 3 mm-maleic acid exerted a distinct inhibitory effect on the uptake by kidney-cortex slices of a variety of naturally occurring amino acids and of the non-utilizable

Table 1. *Effect of the administration of maleic acid on the urinary excretion of free amino acids*

Experimental details are given in the text. 'After maleate' refers to values determined from the first 12 hr. collection of urine after the administration of maleate. Proline, hydroxyproline, leucine, isoleucine and methionine were present in quantities too small to measure.

Amino acid	Rate of excretion (μ g./hr.)							
	Animal 1		Animal 2		Animal 3		Mean	
	Control	After maleate	Control	After maleate	Control	After maleate	Control	After maleate
Taurine	3.6	13.1	4.4	13.7	2.4	12.9	3.4	13.2
Aspartic acid	10.3	10.5	10.5	12.3	8.3	8.6	9.7	10.5
Threonine	24.8	116.9	16.4	82.7	12.9	164.6	18.0	121.4
Serine	11.6	70.0	13.0	49.6	9.4	87.5	11.3	69.0
Asparagine + glutamine	19.9	31.7	30.4	26.9	10.2	56.1	20.2	38.3
Glutamic acid	39.2	216.2	38.0	108.4	32.9	300.4	36.7	208.5
Glycine	41.4	165.7	85.1	126.4	28.9	194.9	51.8	162.3
Alanine	13.0	48.4	14.8	39.7	13.3	52.0	13.7	46.7
Valine	1.6	23.1	4.1	20.7	1.4	34.2	2.4	26.0
Tyrosine	4.6	6.4	9.2	7.6	4.7	10.9	6.2	8.3
Phenylalanine	3.4	6.1	6.9	9.1	3.4	9.9	4.5	8.4
Ornithine	2.7	4.0	3.3	1.3	3.1	11.9	3.0	5.8
Ethanolamine	14.3	16.0	7.9	8.8	12.0	4.7	11.4	9.8
Lysine	36.4	92.2	25.8	121.8	32.8	188.2	31.7	134.1
1-Methylhistidine	3.1	3.0	3.5	—	5.3	5.8	4.0	2.9
Histidine	6.3	24.4	4.8	25.5	5.7	40.6	5.6	30.2
2-Methylhistidine	14.2	8.9	7.4	8.6	9.9	5.8	10.5	7.7
Total	250.3	857.0	285.5	663.1	196.5	1187.0	244.1	903.1

Table 2. *Effect of maleic acid (3 mM) on the uptake of amino acids by kidney-cortex slices*

The experiment was carried out as described in the text. Tissues were incubated in Krebs–Ringer bicarbonate buffer, pH 7.4, at 37° for 60 min. Each flask contained 0.3–0.4 μ C of 14 C-labelled substrate. All values represent the mean of triplicate determinations. The distribution ratio is defined as ratio of 14 C activity/ml. of intracellular fluid to 14 C activity/ml. of medium, and the values represent the means $\pm$ s.e.m. By using Student's *t* test the values for distribution ratio with maleate differed significantly from controls as indicated by *P* values of < 0.02 for each amino acid.

Substrate amino acid	Concn. (μ M)	$10^{-3} \times$ Radioactivity (counts/min./ml.)					
		Medium		Intracellular fluid		Distribution ratio	
		Control	After maleate	Control	After maleate	Control	After maleate
[1- 14 C]AIB	65	247.4	243.3	1016.8	633.5	4.11 $\pm$ 0.08	2.60 $\pm$ 0.16
[2- 14 C]Glycine	170	189.9	183.4	981.3	728.2	5.17 $\pm$ 0.16	3.97 $\pm$ 0.19
L-[U- 14 C]Phenylalanine	75	74.5	69.4	136.7	91.8	1.83 $\pm$ 0.11	1.32 $\pm$ 0.01
L-[U- 14 C]Histidine	95	166.9	166.3	370.1	289.7	2.22 $\pm$ 0.13	1.74 $\pm$ 0.10
L-[U- 14 C]Lysine	60	230.1	235.4	730.2	600.8	3.17 $\pm$ 0.31	2.55 $\pm$ 0.09

Table 3. *Influence of maleic acid concentration on the uptake of α -aminoisobutyric acid and glycine by kidney-cortex slices*

The conditions of incubation were as described in Table 2. Values represent the means of duplicate determinations.

Concn. of maleic acid (mM)	Distribution ratio	
	[1- 14 C]AIB (65 μ M)	[2- 14 C]Glycine (170 μ M)
0	3.82	5.76
0.01	4.30	5.36
0.1	3.45	5.65
0.5	3.52	5.26
1	2.43	4.02
5	2.02	3.76
10	1.80	3.58

Table 4. *Influence of the removal of maleic acid from the medium on the uptake of α -aminoisobutyric acid by kidney-cortex slices*

In the preincubation the tissues were incubated for 45 min. at 37° in Krebs–Ringer bicarbonate buffer, pH 7.4, with or without maleic acid. The tissues were then transferred for incubation to flasks containing [2- 14 C]AIB (65 μ M) in the presence and absence of maleic acid and incubated for 60 min. at 37° in buffer. The values for the distribution ratio represent the means $\pm$ s.e.m. obtained from quadruplicate observations. The distribution ratio is defined in Table 2.

Preincubation conditions	Incubation conditions	Distribution ratio
Buffer	Buffer	4.00 $\pm$ 0.75
Buffer + maleic acid (3 mM)	Buffer	4.23 $\pm$ 0.50
Buffer + maleic acid (3 mM)	Buffer + maleic acid (3 mM)	2.31 $\pm$ 0.06

amino acid AIB. This inhibitory effect was not reversed by pyruvate or glucose (5 mM), nor was it reversed or reproduced by fumaric acid, the natural isomer of maleic acid. D-Malic acid, an intermediate in maleic acid metabolism described

by Taggart, Angielski & Morell (1962), failed to alter the uptake of AIB by kidney-cortex slices. The inhibitory effect of maleate was not seen with concentrations lower than 0.5 mM, but, as shown in Table 3, the maleate effect was concentration-dependent between 0.5 and 10 mM. The concentrations of maleate found to be effective *in vitro* closely approximated the estimated concentration *in vivo*, 6 mM, assuming complete equilibration of the maleate with total body water.

To correlate these observations *in vivo* and *in vitro* further, animals were given single subcutaneous toxic doses of maleic acid and killed 2 hr. later. Kidney-cortex slices from these animals were incubated with AIB, and the uptake by the tissue was compared with that from animals given injections of 0.9 % sodium chloride only. Despite the massive aminoaciduria *in vivo* noted under these conditions, no differences in uptake were found when the kidney-cortex slices were suspended in Krebs–Ringer buffer. However, when kidney-cortex slices from poisoned rats were incubated in plasma from maleate-treated animals, amino acid uptake was distinctly less than that observed when control slices were suspended in control plasma. These discrepant results are explained by the experiments summarized in Table 4, which showed that, when tissues, preincubated in maleic acid for 45 min., were transferred to flasks containing only buffer and labelled AIB, no inhibition of amino acid uptake was observed. This 'wash-out' experiment indicates that the inhibitory effect of maleate on amino acid uptake does not depend on irreversible tissue binding, but rather shows that a significant concentration of maleic acid must be present throughout the incubation to produce inhibition in the system used *in vitro*. Such concentrations are obviously not maintained merely by using tissue from poisoned animals.

Influence of free thiol groups on the effect of maleate in vitro. Morgan & Friedmann (1938b) noted that the free thiol group of glutathione was bound by maleic acid in a preparation of minced liver. Angielski & Rogulski (1959) showed that the amount of free thiol groups of kidney homogenates was decreased within 3 hr. after the administration of maleate, and that animals could be protected from the toxic effects of maleate *in vivo* by the co-administration of 2,3-dimercaptopropanol (BAL). Similar studies *in vitro* were therefore undertaken in the kidney-cortex-slice system, and, as noted in Table 5, equivalent concentrations of mercaptoethanol completely reversed the inhibitory effect of maleate on AIB uptake. Equimolar concentration of thiol-group blockers such as *p*-chloromercuribenzoic acid or *N*-ethylmaleimide were shown to be much more effective inhibitors of AIB uptake than was maleate (Table 6). Further, the effect of *N*-ethylmaleimide could not be 'washed out' by transferring the tissues to inhibitor-free medium.

Michaelis-Menten analysis of the effect of maleic acid on the uptake of α -aminoisobutyric acid by kidney-cortex slices. It has been reported that amino acid uptake by kidney-cortex slices follows Michaelis-Menten kinetics, indicating the presence of saturatable transport sites (Rosenberg *et al.* 1961b; Rosenberg, Downing & Segal, 1962b).

Table 5. *Reversal by mercaptoethanol of the inhibitory effect of maleic acid on the uptake of α -aminoisobutyric acid by kidney-cortex slices*

All tissues were incubated for 120 min. The concentration of [$1\text{-}^{14}\text{C}$]AIB was $65\text{ }\mu\text{M}$. Values represent means $\pm$ S.E.M. derived from quadruplicate observations. The distribution ratio is defined in Table 2.

Experimental conditions	Distribution ratio	Significance of difference from control
Control	3.47 ± 0.40	—
Mercaptoethanol (3 mM)	3.76 ± 0.29	$P > 0.1$
Maleic acid (3 mM)	2.43 ± 0.13	$P < 0.01$
Mercaptoethanol (6 mM) + maleic acid (3 mM)	3.21 ± 0.33	$P > 0.1$

Table 6. *Comparative effects of maleic acid, *p*-chloromercuribenzoate and *N*-ethylmaleimide on the uptake of α -aminoisobutyric acid by kidney-cortex slices*

Tissues were incubated with [$1\text{-}^{14}\text{C}$]AIB ($65\text{ }\mu\text{M}$) for 90 min. Values represent means $\pm$ S.E.M. derived from triplicate observations. The distribution ratio is defined in Table 2.

Inhibitor	Distribution ratio
None	4.55 ± 0.47
Maleic acid (3 mM)	2.74 ± 0.20
<i>p</i> -Chloromercuribenzoate (3 mM)	1.08 ± 0.03
<i>N</i> -Ethylmaleimide (3 mM)	0.89 ± 0.02

Experiments were therefore undertaken to determine whether maleate exerts its inhibitory effect by competing with amino acids for these sites. As shown in Fig. 2, in which the data are plotted according to the method of Lineweaver & Burk (1934), the slope of the plot of the uptake with maleic acid differed markedly from the control, failed to intersect the control plot at the ordinate and extrapolated to a common abscissa value. These findings indicated that maleic acid did not compete with AIB for its saturatable sites.

Kinetic studies of the effect of maleic acid on the uptake of α -aminoisobutyric acid by kidney slices. Steady-state kinetic studies were performed to determine whether maleic acid inhibited the uptake of amino acids by slices by selectively altering influx or efflux. In these experiments slices were incubated in unlabelled amino acid until steady-state conditions were reached, at which time tracer quantities of [$1\text{-}^{14}\text{C}$]AIB were added. The time-curves obtained are shown in Fig. 3. These data were subjected to analysis by using the multi-

Fig. 2. Lineweaver-Burk plot of the effect of maleic acid (3 mM) on the uptake of [$1\text{-}^{14}\text{C}$]AIB by kidney-cortex slices. v represents the velocity of uptake by the saturatable system. The common abscissa intercept indicates non-competitive inhibition by maleic acid. Each point is an average of triplicate determinations. ●, AIB control; ○, AIB + maleic acid (3 mM).

compartment model system that has been described for the kidney-cortex slice (Rosenberg, Berman & Segal, 1963), based on the model approach and digital-computer techniques of Berman, Weiss & Shahn (1962) and Berman, Shahn & Weiss (1962). This analysis revealed that the transport of amino acids by kidney-cortex slices may be satisfied by a three-compartment parallel model as shown in Scheme 1. (A series model

representing amino acid movement from media to extracellular and then into intracellular fluid was incompatible with the kinetic data.) The effect of maleic acid on the parameters of this model (Scheme 1) revealed that maleate not only decreased the cellular influx of amino acid but to a greater extent increased the efflux. Similar results have been reported with 2,4-dinitrophenol (Rosenberg *et al.* 1963).

Failure of maleate to affect the uptake of amino acids by gut and muscle. To determine whether the influence of maleic acid on transport mechanisms is specific for the kidney, uptake studies were performed with diaphragm muscle and everted jejunal segments. At concentrations capable of producing distinct inhibition of amino acid uptake by kidney-cortex slices, maleate failed to alter the uptake by muscle of AIB or the uptake by gut of glycine, L-lysine or 1-aminocyclopentane-1-carboxylic acid (cycloleucine), another non-metabolizable model amino acid studied by Akedo & Christensen (1962).

Uptake and oxidation of [2,3- ^{14}C]maleic acid by rat tissues in vitro. Studies of the uptake of [2,3- $^{14}\text{C}_2$]maleic acid were undertaken in preparations of gut, kidney and muscle to determine whether the specificity of maleic acid for kidney tissue depended on its ability to enter only that tissue. Table 7 summarizes the results of such uptake studies, indicating that, in contrast with kidney-cortex slices where the radioactivity of the intracellular fluid approximated that in the medium (ratios of about 1.0), gut and muscle cells were essentially impermeable to maleate. To correlate further the effects produced by maleic acid with its metabolism, experiments were carried out

Fig. 3. Time-course of the inhibition by maleic acid (3 mM) of the uptake of [1- ^{14}C]AIB by kidney-cortex slices. Ordinate values are expressed as uptake of fraction of initial medium AIB concentration/100 mg. of tissue. The calculated points represent least-squares fitting of the observed values obtained with the compartmental system described by Rosenberg, Berman & Segal (1963). Steady-state distribution ratios were 4.55 for the control and 2.55 for maleate-containing flasks. Each point represents the mean of triplicate determinations. ●, Control, observed; ■, maleic acid, observed; ○, control, calculated; □, maleic acid, calculated.

Scheme 1. Kinetics of the uptake of [1- ^{14}C]AIB by kidney-cortex slices in the presence and absence of maleic acid (3 mM). The data shown in Fig. 3 were analysed according to the three compartment model system shown, and appropriate rate constants calculated. The extracellular-space kinetics, solved with ^{14}C -labelled inulin, were unchanged by maleate. The numerical values represent rate constants (min^{-1}) in the directions noted by the arrows. Maleic acid decreased the steady-state intracellular amino acid pool by accelerating efflux and, to a lesser degree, by retarding influx.

Table 7. Penetration of [2,3-¹⁴C₂]maleic acid into kidney-cortex slices, gut segments and diaphragm muscle

Tissues were incubated for 60 min. with a tracer concentration (0.1 μc/ml.) of [2,3-¹⁴C₂]maleic acid. Triplicate determinations were averaged. Numerical values represent means ± s.e.m. The distribution ratio is defined in Table 2.

Tissue preparation	Distribution ratio
Kidney slices	1.13 ± 0.08
Gut segments	0.28 ± 0.04
Diaphragm muscle	<0.1

Table 8. Oxidation of [2,3-¹⁴C₂]maleic acid to ¹⁴CO₂ by rat tissues in vitro

Tissues were incubated for 90 min. with a tracer concentration (0.2 μc/flask) of [2,3-¹⁴C₂]maleic acid. Values represent means of triplicate determinations.

Tissue preparation	¹⁴ CO ₂ produced	
	(counts/min./100 mg. of tissue)	(% of initial dose/100 mg. of tissue)
Diaphragm muscle	2405	1.08
Gut segments	3070	1.38
Liver slices	7880	3.53
Spleen slices	1219	0.55
Kidney slices	20400	9.19

to measure the extent of ¹⁴CO₂ evolution from [2,3-¹⁴C₂]maleic acid by various rat tissues (Table 8). As expected from the uptake data, gut and muscle tissue metabolized very little of the labelled maleate, as did spleen and liver. In contrast, kidney-cortex slices oxidized more than 9 % of the initial maleate to ¹⁴CO₂ during 60 min. incubation studies. While these investigations were in progress, Taggart *et al.* (1962) reported findings which these results confirm.

DISCUSSION

The findings *in vivo* reported above are in complete agreement with previous work, all clearly indicating that maleic acid exerts a profound toxic effect on a multiplicity of renal tubular transport mechanisms. Yet the biochemical mechanism of this effect remains unanswered. Two general types of action deserve mention: a direct effect of maleate on the membrane of the cells lining the renal tubule; or an interference with intracellular metabolic processes that supply energy to active transport systems in the renal tubular cells. The data from amino acid-uptake experiments in kidney-cortex slices, showing an inhibitory effect of maleic acid, correlate very well with the observations *in vivo*, but unfortunately provide only indirect evidence for a locus of the toxic effects. The failure of maleic acid to compete with AIB or to

alter the affinity of this model substrate for its saturatable binding site suggests that the mechanism of action does not reside solely in the membrane. Further, kinetic analysis of the inhibition by maleic acid revealed a decrease in influx and an acceleration of efflux. These findings reproduce those previously reported with 2,4-dinitrophenol, a substance known to interfere with oxidative phosphorylation. Although these observations imply that maleic acid alters membrane transport secondarily by interfering with intracellular metabolic processes, the possibility exists that maleate may act by both of the general mechanisms described above. It is of note that Worthen (1963), using the electron microscope, reported that maleic acid-poisoned rats showed shortening of the proximal tubular-cell epithelial brush border as well as intracellular vacuolation and distortion of the basal portion of tubular cells. No glomerular or distal tubular abnormalities were seen.

The work of Angielski & Rogulski (1959) has emphasized the relationship between the known effect of maleate as a binder of free thiol groups and its toxic effect on the kidney. Our data with mercaptoethanol indicate that the toxic effect of maleate can be reversed by providing an adequate concentration of free thiol groups to combine with the maleic acid and thus prevent it from being reactive. But these findings in no way prove that this avidity for free thiol groups has anything to do with the mechanism of the maleate effect. On the contrary, the 'wash-out' experiment reported above, in which maleate was shown not to be firmly bound to the kidney-cortex slices, argues against a covalent link between maleate and cellular thiol groups as the mechanism of its action. If, as the data indicate, the toxic effect of maleate is correlated with the ability of the tissue to metabolize this compound, the reversal of the toxicity by mercaptoethanol might be explained by an inability of the addition product of the thiol with maleate to undergo the same metabolic reactions as maleate itself. Perhaps even more significant is the fact that, during the formation of addition compounds of maleate with thiols, much of the toxic maleic acid is converted into fumaric acid (Morgan & Friedmann, 1938*a*). Worthen (1963) reported a distinct decrease in succinate-dehydrogenase activity in kidney from maleate-poisoned rats, but found no decrease in kidney thiol content.

The singular effect of maleic acid on the uptake of amino acids by the kidney appears to be due, at least in part, to differences in permeability between kidney-cortex-slice cells and those of gut segments or diaphragm muscle. It seems likely that maleic acid fails to interfere with transport phenomena in gut and muscle because it fails to penetrate the cells of these tissues and be metabolized, as it is in

the kidney. Although D-malic acid, a known intermediate in maleic acid metabolism, failed to affect amino acid uptake by kidney-cortex slices, further investigation of maleic acid metabolism is needed to determine whether the deleterious effects of this substance result from one of its metabolic products or reside solely in the intact molecule. The correlation between tissue permeability, metabolism and the toxic effects of maleic acid provide additional evidence for an intracellular site of action of this drug.

The striking similarity between the effects of maleic acid and the constellation of tubular defects commonly referred to as 'the Fanconi syndrome' is worthy of mention. A great many disease processes and specific poisoning processes have been shown to produce a similar picture of glycosuria, amino-aciduria, phosphaturia and variable effects on electrolyte excretion and urinary acidification (Leaf, 1960), but there is little information available on the mechanism of these effects. It seems reasonable that the similarity of tubular defects resulting from such a wide range of aetiological agents is related to a common interference with energy-yielding reactions needed by active transport systems rather than to specific effects on the renal tubular-cell membrane. In contrast, isolated defects such as renal glycosuria (Joslin, Root, White & Marble, 1940), glycinuria (DeVries, Kochwa, Lazebnik, Frank & Djaldetti, 1957) or cystinuria (Knox, 1960) may well result from specific structural or enzymic defects in the cell membrane.

SUMMARY

1. The previously reported deleterious effects of the administration of maleic acid *in vivo* on renal amino acid transport have been extended to studies *in vitro* in which maleic acid markedly inhibited amino acid uptake by kidney-cortex slices.

2. This inhibition has been observed at concentrations which approximate those obtained *in vivo* and is due to accelerated efflux and impaired influx of amino acids in kidney cells.

3. The results showed that maleate does not compete for a common transport site with amino acids but rather suggested that it alters renal tubular transport by interfering with intracellular processes essential to active amino acid transport. The studies failed to support previous investigations relating the mechanism of maleate action to the binding of free thiol groups.

4. The specificity of the toxic effects of maleic acid for renal transport systems was shown to be due, at least in part, to its ability to penetrate kidney cells, and its failure to enter muscle or intestinal cells.

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