Amino Ketone Synthesis in Avian Erythrocytes

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1. The relative rates of synthesis of aminolaevulate and aminoacetone by particles prepared from avian erythrocytes were measured under various conditions. 2. The production of both amino ketones by fresh particles was about three times greater in anaemia caused by phenylhydrazine and acetylphenylhydrazine than in anaemia caused by removal of 20–30 ml. of blood. 3. The synthesis of aminolaevulate by freeze-dried particles decreased more than that of aminoacetone in the absence of added pyridoxal phosphate, in the presence of cyanide and of tris buffer, and after preincubation of the erythrocyte particles. Other differences in the rates of synthesis of the two amino ketones were observed after (a) incubation of particles at different temperatures and (b) storage of homogenized freeze-dried particles at different pH values. 4. It is suggested that these differences in the production of the two amino ketones are due to the presence of two amino ketone synthetases or to two or more isoenzymes of aminolaevulate synthetase. 5. The metabolic significance of aminoacetone in erythrocytes is discussed.

Aminolaevulate and aminoacetone are produced by avian erythrocytes (Gibson, Laver & Neuberger, 1958), micro-organisms (Elliott, 1958; Gibson, 1958; Kikuchi, Kumar, Talmage & Shemin, 1958) and liver mitochondria (Urata & Granick, 1961; Granick & Urata, 1963; Tschudy, Welland, Collins & Hunter, 1964). The formation of other amino ketones by erythrocyte particles (Gibson *et al.* 1958), extracts of *Rhodospseudomonas spheroides* (Kikuchi, Kumar & Shemin, 1959) and liver mitochondria (Urata & Granick, 1963) suggested that δ -aminolaevulate synthetase has a wide specificity. However, several drugs produce a large increase in the formation of aminolaevulate by liver mitochondria but fail to affect aminoacetone production, and this suggests that there are at least two amino ketone synthetases in liver (Granick & Urata, 1962).

In view of the apparent differences in the properties of amino ketone synthetases in different tissues, the formation of aminolaevulate and aminoacetone was re-examined in particles obtained from avian erythrocytes.

MATERIALS AND METHODS

Reagents. The following substances were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.: CoA, 5'-pyridoxal phosphate, DL-penicillamine, L-cysteine, δ -aminolaevulinic acid, 2-oxoglutarate, acetylphenylhydrazine and succinic anhydride. Sodium pyruvate, iodoacetamide, L-penicillamine and *p*-chloromercuribenzoate were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Peroxide-free diethyl ether was from May and Baker Ltd.,

Dagenham, Essex. Haemin was from Fluka A.-G. Chemische Fabrik, Buchs, Switzerland. Aminoacetone hydrochloride was a gift from Professor A. Neuberger of St Mary's Hospital, London W.2. Sodium pyruvate and *p*-dimethylaminobenzaldehyde were recrystallized from ethanol. Ehrlich's reagent, which contained HgCl_2 , was made up by the procedure of Urata & Granick (1963). Phosphate buffer, pH 7.3, was made from KH_2PO_4 and K_2HPO_4 . Haemin solutions (1.5 mM) were made up as suggested by Burnham & Lascelles (1963), except that *m*-phosphate buffer, pH 7.3, was used instead of *m*-tris-HCl buffer. The other inhibitors were made up in aqueous solution. Succinyl-CoA and acetyl-CoA were each prepared from CoA and the corresponding anhydride (Simon & Shemin, 1953); *m*-HCl was then added until the pH reached 2–3 and the solution was stored in 2 ml. portions at -18° .

Peroxide-free ether was equilibrated with an aqueous solution containing, in 300 ml., the following reagents (in *m*-moles): trichloroacetic acid, 55; sodium acetate, 72; NaOH, 19; KCl, 9.2; Na_2HPO_4 , 3.3; K_2HPO_4 – KH_2PO_4 buffer (pH 7.3), 7.4. The final pH of the solution was 4.85. Equilibration was carried out by shaking equal volumes of this solution and ether at room temperature for 5 min.

Erythrocyte particles

The domestic hen (*Gallus domesticus*) was used as the source of erythrocyte particles. Most of the birds were laying hens 18–24 months old, but one hen, 7 years old, and one cockerel, 2 years old, were used also. The birds were made anaemic to increase the capacity of the particles to synthesize amino ketones. This was carried out by either (a) removal of 20–30 ml. of blood from a wing vein under ether anaesthesia 4 days before preparation of the particles or (b) intramuscular injection, in a common solution at pH 7.3,

of 25 mg. of phenylhydrazine and of acetylphenylhydrazine/kg. 65–67 hr. before preparation of the particles (Laver, Neuberger & Udenfriend, 1958). The amounts of the hydrazines injected were varied slightly in a few experiments, but there was no obvious relation between the dose and the production of amino ketones by the erythrocyte particles. Attempts to increase the dose by more than 10% usually killed the birds. The degree of anaemia produced by the mixed hydrazines varied considerably even with a constant dose, and is presumably related to the haemoglobin concentration before treatment.

Collection of blood. Each hen was anaesthetized with ether and injected with 8000 i.u. of heparin through a wing vein. After 3–4 min. the head was cut off, the cut ends of the oesophagus and trachea were compressed manually and the blood was collected in a beaker standing in melting ice. About 75 ml. of blood was obtained from each bird.

Preparation of particles. The method used was that of Laver *et al.* (1958). Each preparation occupied 5–6 hr. The two layers described by Laver *et al.* (1958) were observed in preparations made from birds treated with the mixed phenylhydrazines and the top layer of particles only was used. However, in preparations obtained from birds made anaemic by bleeding, three well-defined layers were noticed: (a) a deposit rich in haemoglobin, erythrocytes, or both, and small clots, occupying 10–15% of the total volume, (b) a middle viscous layer occupying about 70% of the total volume and coloured yellow–brown in the final preparation, and (c) a top layer of pink particles of low viscosity, comprising about 15% of the total volume. The lowest layer together with the lowest fraction of the middle layer were discarded after each washing of the preparation. The final preparation contained no visible haemoglobin and comprised the top layer and about 75% of the middle layer. In two preparations made from birds given small doses of the mixed phenylhydrazines three layers were again observed, the middle layer being brown. Microscopy of the preparation showed that both middle and top layers consisted chiefly of nuclei and that the middle layer contained more cytoplasm in which the nuclei were aggregated.

Freeze-drying of particles. Fresh particles were frozen rapidly in acetone–solid CO₂ mixture and freeze-dried by Glaxo Laboratories Ltd., Greenford, Middlesex. They were then stored at –18° until required (Laver *et al.* 1958). They were prepared for use by suspension in water at 0° (approx. 100 mg./3 ml.) and homogenization in a Potter–Elvehjem homogenizer for 3 min. One-third of the weight of the freeze-dried ‘particles’ used in experiments was due to the KCl required for their preparation.

Synthesis of amino ketones from fresh particles

Erythrocyte particles (1–2 hr. old) were incubated as indicated in Table 1 (Laver *et al.* 1958; Gibson *et al.* 1958). After the reaction had been stopped by addition of trichloroacetic acid, the supernatant containing the amino ketones was removed by centrifuging and stored at –18° for 12–36 hr. until required.

Determination of aminoacetone and aminolaevulate in a common solution. The separation and determination of the amino ketones was carried out by the method of Granick (1966), in which the aminoacetone-pyrrole was extracted from the aqueous supernatant at pH 7.3 into ether equilibrated against an aqueous solution at pH 4.85 and of similar

composition to that containing the two amino ketones (see under ‘Reagents’ above for preparation of equilibrated ether). Under these conditions 85% of the aminolaevulate-pyrrole and 5% of the aminoacetone-pyrrole remained in the aqueous phase. The quantities of the reagents used by Granick (1966) to prepare the supernatant for partition were modified to allow for the slightly different conditions of incubation and protein precipitation. Thus the pH of the supernatant obtained after incubation was adjusted to 4.6 with 0.55 ml. of 1.6 M-sodium acetate and the 2 ml. of solution from which aminoacetone-pyrrole was extracted was adjusted to pH 7–7.5 by the addition of 0.05 ml. of 0.5 M-Na₂HPO₄ and 0.2 ml. of 1.0 M-NaOH. The KClO₄ that formed from the KCl in the reaction mixture and the HClO₄ of the Ehrlich’s reagent was spun down and the supernatant was removed. The E_{552} of the aqueous solution containing the Ehrlich colour salt of the pyrroles of both amino ketones was measured before partition (W) and after partition (Z). The extinctions were converted into molar quantities by using ϵ_{552} 5.8×10^4 and 6.6×10^4 for the Ehrlich colour salts of aminolaevulate- and aminoacetone-pyrroles respectively. The amounts of the amino ketones present in the original 1.5 ml. of the incubation mixture were calculated by solving two simultaneous equations relating the different percentages of the two amino ketone pyrroles present in the aqueous phase before and after partition. The equations used differ slightly from Granick’s (1966) because of the different volumes of neutralizing solutions used, as indicated above.

$$\text{Moles of aminolaevulate} = 1.7 \times 10^{-7}(Z - 0.0445W)$$

$$\text{Moles of aminoacetone} = 1.49 \times 10^{-7}(0.756W - Z)$$

Determination of aminolaevulate. A suitable sample of the supernatant (adjusted to pH 4.6, as above) was diluted to 1.0 ml. with 0.05 M-sodium acetate, pH 4.6. Aminolaevulate was then determined by the method of Granick (1966) except that the KClO₄ formed (see above) was spun down and the supernatant was used to measure E_{552} . The amount of aminolaevulate present was calculated from ϵ_{552} 5.8×10^4 .

Synthesis of amino ketones from freeze-dried particles

The two amino ketones were synthesized separately in the medium indicated in Table 4 (Gibson *et al.* 1958), succinyl-CoA being used for aminolaevulate synthesis and acetyl-CoA for aminoacetone synthesis. After the reaction had been stopped with trichloroacetic acid the supernatant was removed and adjusted to pH 4.6 by the addition of 0.2 ml. of 1.6 M-sodium acetate. Then 1.0 ml. of this solution was removed and used for determination of the amino ketone content (Granick, 1966). The formation of KClO₄ was avoided by using a sodium phosphate buffer for the enzyme reaction, and by making up the volume of the reaction mixture with water instead of KCl solution. The amounts of the amino ketones formed were calculated from the extinction coefficients already given.

RESULTS

Experiments with fresh particles

The amounts of aminolaevulate and aminoacetone produced by the erythrocyte particles

Table 1. *Production of amino ketones from fresh erythrocyte particles*

The reaction mixture contained erythrocyte particles (0.1–0.9 ml.) together with the following reagents (in μ moles): glycine, 90; $MgCl_2$, 4.5; K_2HPO_4 – KH_2PO_4 (pH 7.3), 100; CoA, 0.07; pyridoxal phosphate, 0.20; potassium oxoglutarate, 4.5, or sodium pyruvate, 4.5. KCl solution (154 mM) was used to make up the volume to 1.5 ml. The tubes were lightly stoppered with water-repellent cotton wool and were incubated in air at 37° for 90 min., being shaken 130 times/min. The reaction was stopped by adding 1.5 ml. of 8% (w/v) trichloroacetic acid. The amounts of the amino ketones produced are expressed in nmoles/ml. of packed particles/90 min. at 37°. Average values are given with either individual values or the ranges of values in parentheses.

State of hen	No. of expts.*	Aminolaevulate		Amino acetone From pyruvate	Ratio $\frac{\text{aminolaevulate}}{\text{aminoacetone}}$	
		From oxoglutarate	From pyruvate		aminolaevulate from oxoglutarate	
					Ratio $\frac{\text{aminoacetone from pyruvate}}{\text{aminoacetone from pyruvate}}$	From pyruvate
Normal or bled†	3	100 (68, 84, 150)	—	—	—	—
Bled†	3	90 (68, 106, 96)	6 (7, 8, 4)	16 (16, 17, 15)	5.7 (4.3, 7.0, 5.7)	0.41 (0.43, 0.53, 0.27)
Anaemic from mixed phenylhydrazines‡	9	324 (186-486)	22 (14-32)	44 (26-58)	7.4 (5.6-10.8)	0.49 (0.35-0.65)

* Blood from one hen was used for each experiment.

† Results refer to the middle layer of three layers of particles.

‡ Results refer to the top layer of two layers of particles in seven experiments where the dose of each phenylhydrazine was 25 mg./kg., to the top layer of three layers in one experiment where the dose was 12 mg./kg. and to the middle layer of three layers of particles in one experiment where the dose was 16 mg./kg.

Table 2. *Production of amino ketones from top and middle layers of fresh erythrocyte particles*

The conditions used are given in Table 1. The amino ketone production is expressed as

$$\frac{(\text{amount produced by top layer})}{(\text{amount produced by middle layer})} \times 100\%.$$

Expt. no.	Treatment of hen	Time between treatment and preparation of particles (hr.)	Aminolaevulate		Aminoacetone From pyruvate
			From oxoglutarate	From pyruvate	
1	Bled of 20–30 ml.	91	34	17	45
2	Bled of 20–30 ml.	91	31	43	33
3	Bled of 20–30 ml.	91	19	33	39
4	Phenylhydrazine + acetylphenylhydrazine (12 mg./kg.)	68	129	113	151
5	Phenylhydrazine + acetylphenylhydrazine (16 mg./kg.)	91	68	64	70

obtained from birds made anaemic by bleeding and by injection of mixed phenylhydrazines are shown in Table 1. The composition of the reaction medium and the conditions of incubation are given with the table. With oxoglutarate as substrate only aminolaevulate was produced, but with pyruvate both metabolites were formed. The synthesis of the amino ketones was about three times as great, from both substrates, by particles obtained from birds made anaemic by the mixed phenylhydrazines as that by normal birds or those that were bled. With pyruvate, the ratio (aminolaevulate produced)/

(aminoacetone produced) was about 0.45, and the ratio (aminolaevulate produced from oxoglutarate)/(aminoacetone produced from pyruvate) was about 6.5.

The relative rates of synthesis of the two amino ketones from the top and middle layers (see under 'Preparation of particles' in the Materials and Methods section) are given in Table 2. (a) Particles from birds made anaemic by bleeding. The production of both amino ketones by the top layer varied from 17% to 45% of the production by the middle layer. When particles from the two layers

were mixed the amounts of amino ketones produced were equal to the sum of those produced by the separated layers. (b) Particles from birds made anaemic by the mixed phenylhydrazines. When 25mg. of each hydrazine/kg. was injected, only one layer was formed above the bottom layer of clots and debris. However, in two experiments where 12mg. and 16mg. of each hydrazine/kg. were injected the top and middle layers were observed. The 12mg./kg. dose followed by exsanguination at 68hr. was associated with a greater production of the amino ketones from the top layer, whereas after the 16mg./kg. dose followed by exsanguination at 91hr. the middle layer was the more active.

Table 3. *Effects of inhibitors on amino ketone production by fresh erythrocyte particles*

The top layer of particles obtained from birds treated with mixed phenylhydrazines was used. The conditions were the same as in Table 1, except that inhibitors were added to give the concentrations shown. The results are expressed as nmoles/ml. of packed particles/90 min.

Inhibitor	Activity remaining (%)		
	Aminolaevulate		Aminoacetone
	From oxoglutarate	From pyruvate	From pyruvate
DL-Penicillamine (13mM)	15	40	20
L-Cysteine (27mM)	10	40	20
KCN (5mM)	3	56	*
FeSO ₄ (1mM)	73	—	—
Haemin (100μM)	81	—	—
(10μM)	100	103	100

* In four experiments the activity remaining was 110, 55, 96 and 60%.

Omission of pyridoxal phosphate failed to affect aminoacetone formation in three out of four experiments, whereas this omission caused aminolaevulate formation from both oxoglutarate and pyruvate to decrease by 15–33%.

The effects of inhibitors on amino ketone formation are shown in Table 3. Both DL-penicillamine and L-cysteine were inhibitory. Cyanide inhibited the formation of aminolaevulate from oxoglutarate strongly and from pyruvate moderately; the effect on aminoacetone production was variable. Fe²⁺ ions (1mM) inhibited aminolaevulate synthesis by 25–30%. Haemin (100μM) decreased aminolaevulate synthesis by about 20%, but had no effect on the synthesis of either of the amino ketones at 10μM.

Experiments with freeze-dried particles

Optimum conditions. The amounts of aminoacetone and aminolaevulate produced by five particle preparations are shown in Table 4. In preparation 3 the freeze-drying was incomplete, and this was associated with diminished amino ketone formation; also, the ratio (aminoacetone produced)/(aminolaevulate produced) fell from 72% in the first week of storage to 30% in the fourth week. In preparation 5, where an attempt was made to obtain a larger yield of light particles by not removing all of the heavy deposit, the synthesis of aminoacetone was greatly diminished.

The effect on amino ketone production of increasing the weight of particles added from 0.8 to 5mg./tube is shown in Fig. 1. The curve for effect of glycine concentration appears in Fig. 2, which shows that at about 60mM-glycine the production of aminolaevulate reached a plateau whereas the production of aminoacetone was still rising. The synthesis of both amino ketones increased to the

Table 4. *Production of amino ketones from freeze-dried erythrocyte particles*

The reaction mixture contained 2–6 mg. of erythrocyte particles together with the following reagents (in μmoles): glycine, 45; Na₂HPO₄–NaH₂PO₄ (pH 7.4), 50; pyridoxal phosphate, 0.1; acetyl-CoA or succinyl-CoA, 0.18. The volume was made up to 0.75 ml. with water. The tubes were incubated open in air at 37° for 30 min., being shaken 130 times/min. The reaction was stopped by adding 0.5 ml. of 10% (w/v) trichloroacetic acid. The results are expressed as nmoles of amino ketone produced/30 mg. of particles/30 min. at 37°.

Preparation	Aminolaevulate from succinyl-CoA	Aminoacetone from acetyl-CoA	Ratio $\frac{\text{aminoacetone}}{\text{aminolaevulate}}$ (%)
1*	100	48	48
2	180	75	42
3†	50	36	72
4	156	64	41
5‡	178	15	8

* Particles homogenized for 1 min. only and volume of reaction mixture made up with 0.25 ml. of 155 mM-KCl.

† Freeze-drying incomplete: three to four times as many particles used for each assay.

‡ Active particles incompletely freed from brown deposit during preparation.

Fig. 1. Effect of amount of particles added on production of amino ketones. The conditions used are given in Table 4. ●, Aminolaevulate; ▲, aminoacetone.

Fig. 2. Effect of concentration of glycine on production of amino ketones. The conditions used are given in Table 4. ●, Aminolaevulate; ▲, aminoacetone.

Fig. 3. Effect of added acyl-CoA on production of amino ketones. The conditions used are given in Table 4. ●, Aminolaevulate; ▲, aminoacetone.

buffers (Table 5). Addition of 100 and 400 μ moles of potassium chloride to the reaction mixture depressed the formation of both amino ketones to about 70% and 30% respectively of control values. Increasing the temperature of incubation from 37.5° to 47° decreased the formation of aminolaevulate to 70% of control values but increased that of aminoacetone to 130%.

Effect of storage of homogenized particles (Table 6). Storage in water alone decreased the production of both amino ketones by the particles. Storage in phosphate buffer at pH 6.01 had little effect on the production of aminoacetone and caused a decrease in aminolaevulate production, whereas storage at pH 7.42 caused a decrease in aminoacetone formation and had a small and variable effect on aminolaevulate formation. The addition of glycine and pyridoxal phosphate to the storage medium had no more effect than phosphate buffer alone at pH 7.42. Storage in tris buffer at pH 9.00 resulted in a moderate decrease in aminolaevulate synthesis and a complete cessation of aminoacetone synthesis.

Effect of physical stress on homogenized particles (Table 7). Preincubation of the aqueous homogenate with or without buffer decreased aminolaevulate synthesis, but in the presence of the complete reaction mixture minus succinyl-CoA no decrease in the rate of synthesis occurred. Aminoacetone production was unaffected by preincubation at 37° and 40% of the control value remained

same extent when the concentration of the relevant acyl-CoA was increased (Fig. 3). Omission of pyridoxal phosphate from the reaction mixture decreased the production of aminolaevulate and aminoacetone to about 50% and 90% respectively of the control values. The optimum pH for aminolaevulate formation in both phosphate and tris buffers was 7.4. The optimum pH for aminoacetone formation in both of these buffers was 7.7, the reaction being 5–10% slower at pH 7.4. The production of aminolaevulate in tris buffer was about 75% of the production in phosphate buffer whereas the production of aminoacetone was the same in both

Table 5. *Effect of buffer on production of amino ketones by erythrocyte particles*

The conditions were the same as in Table 4, except (a) the pH of the buffers used is given below, that for tris-HCl (67 mM) being converted into the value at 37°, and (b) in Expts. 1 and 2 the volume of the reaction mixture was made up to 0.75 ml. with 154 mM-KCl.

Aminolaevulate				Aminoacetone			
Expt. no.	Buffer	Amount formed		Buffer	Amount formed		
		nmoles/30 mg. of particles/ 30 min.	tris-HCl phosphate ratio (%)		nmoles/30 mg. of particles/ 30 min.	tris-HCl phosphate ratio (%)	
1	Phosphate, pH 7.50	100	68	Phosphate, pH 7.80	52	106	
	Tris-HCl, pH 7.55	68		Tris-HCl, pH 7.76	55		
2	Phosphate, pH 7.50	132	83	Phosphate, pH 7.70	54	113	
	Tris-HCl, pH 7.76	110		Tris-HCl, pH 7.76	61		
3	Phosphate, pH 7.42	189	72	Phosphate, pH 7.42	77	92*	
	Tris-HCl, pH 7.55	135		Tris-HCl, pH 7.55	71		
4	Phosphate, pH 7.42	50	68	Phosphate, pH 7.42	72	89*	
	Tris-HCl, pH 7.55	34		Tris-HCl, pH 7.55	64		

* The lower percentage in these experiments is due to the relatively low pH of the phosphate buffer.

Table 6. *Effect of storage of homogenized erythrocyte particles at 0° on amino ketone production*

Erythrocyte particles were homogenized in water as usual; those required for the control experiments were used immediately and the remainder were stored at 0° in the media indicated for 24 hr. The conditions of incubation are given in Table 4. The results are expressed as nmoles of amino ketone produced/30 mg. of particles/30 min.

Storage conditions			Aminolaevulate			Aminoacetone		
			test	control	test	test	control	test
ratio (%)	control	ratio (%)						
Buffer	pH	test	control	ratio (%)	test	control	ratio (%)	
Water*	—	17	31	55	9	16	56	
Phosphate (0.033 M)								
Expt. 1	6.01	56	118	47	46	49	94	
	6.57	74	118	63	45	49	92	
	7.42	91	118	77	30	49	61	
	7.42†	—	—	—	29	49	59	
Expt. 2	6.57	102	124	82	52	59	89	
	7.42	134	124	108	33	59	56	
	7.42‡	118	124	95	39	59	67	
Tris-HCl (0.033 M)	7.58	102	124	82	8	59	13	
	9.00	83	124	67	0	59	0	

* Stored at 0° for 48 hr.

† 2.5 mg. of particles stored in 0.6 ml. containing buffer, pyridoxal phosphate (0.1 μmole) and glycine (45 μmoles).

‡ 13 mg. of particles stored in 0.7 ml. containing buffer, pyridoxal phosphate (0.08 μmole) and glycine (36 μmoles).

even after preincubation at 55°. Rapid freezing and thawing of the particles had little effect on amino ketone production. Sonication appeared to produce a slight decrease in aminolaevulate formation and a small increase in aminoacetone formation.

Effect of various substances on amino ketone production (Table 8). Cyanide almost completely inhibited aminolaevulate production at a concentration of 50 μM, whereas aminoacetone production was little, if any, affected at 5 mM. CoA and L-penicillamine inhibited the formation of both

Table 7. *Effect of physical stress on amino ketone production by erythrocyte particles*

Erythrocyte particles were homogenized in water. In Expts. 1-4 they were preincubated for the times and at the temperatures shown. In Expts. 5-7 the particles were frozen in acetone-solid CO₂ and immediately thawed at 37°. In Expt. 8 the homogenized particles were put into a deep-freeze at -18° and after 48 hr. they were allowed to thaw at room temperature. In Expt. 9 sonication was carried out with an MSE ultrasonic disintegrator at 1.2A with a 9 mm. probe. Otherwise the conditions used were as in Table 4. The results are expressed as nmoles of amino ketone produced/30 mg. of particles/30 min.

Expt. no.	Conditions	Aminolaevulate			Aminoacetone		
		test	control	test	test	control	test
				control ratio (%)			control ratio (%)
1	37°, 10 min.	75	112	67	66	54	122
	37°, 30 min.	51	112	46	55	54	102
	37°, 30 min.*	109	112	97	54	54	100
	37°, 30 min.†	48	118	41	—	—	—
2	45°, 10 min.	31	172	18	7	10	70
	55°, 10 min.	13	172	8	4	10	40
3	50°, 10 min.	4	33	12	17	18	95
4	50°, 10 min.	28	156	18	49	64	77
5	1 freeze-thaw, rapid	30	33	91	19	18	109
6	3 freeze-thaws, rapid	162	156	104	77	64	121
7	5 freeze-thaws, rapid	136	150	91	64	59	109
8	1 freeze-thaw, slow	18	31	58	10	16	64
9	Sonication: 2 min.	161	172	94	11	10	110
	4 min.	140	172	81	12	10	120

* The particles were preincubated in the normal reaction medium (see Table 4) minus acyl-CoA.

† The particles were preincubated in 100 mM-phosphate buffer, pH 7.42.

amino ketones, aminoacetone formation being more affected. L-Cysteine (results not given in Table 8) inhibited the production of both amino ketones to the same extent as L-penicillamine affected aminolaevulate production. Fe²⁺ ions inhibited aminolaevulate synthesis at all concentrations used but decreased aminoacetone synthesis only at 5 mM. Haemin (100 μM) had approximately the same effect as Fe²⁺ ions (100 μM) on aminolaevulate production but did not affect aminoacetone production; at 600 μM haemin was a more potent inhibitor than Fe²⁺ ions and inhibited the formation of aminolaevulate and aminoacetone to about the same extent. There were no differences in the effects of the other substances tested on the formation of the two amino ketones. EDTA (results not shown in Table 8) did not inhibit the synthesis of either amino ketone at 1 mM, nor did it affect synthesis of aminoacetone at 10 mM.

DISCUSSION

Evidence for more than one amino ketone synthetase. The relatively small change in the ratio of

the amounts of aminoacetone and aminolaevulate formed over a threefold range of production could be accounted for by the broad specificity of a single enzyme. However, the different effects of chemical and physical agents on the production of aminoacetone and aminolaevulate suggest that there are separate synthetases catalysing the formation of each amino ketone.

The requirement for pyridoxal phosphate is greater for the formation of aminolaevulate than for that of aminoacetone. The greater requirement for pyridoxal phosphate by freeze-dried particles than by fresh particles during aminolaevulate synthesis suggests that the binding of the cofactor to aminolaevulate synthetase is not sufficiently strong to prevent losses during freeze-drying, rehydration, or both, of the particles.

Many of the effects of various modifiers (Tables 3 and 8) on the production of aminoacetone and aminolaevulate could be explained on the basis of a weaker aldimine bond being formed between pyridoxal phosphate and a synthetase specific for the formation of aminolaevulate. The inhibition by both cyanide and tris buffer could be accounted for

Table 8. *Effect of various substances on amino ketone production by erythrocyte particles*

The conditions were the same as in Table 4, except for the addition of the substances shown. The results are expressed as nmoles of amino ketone produced/30 mg. of particles/30 min.

Additions	Activity remaining (%)	
	Aminolaevulate	Aminoacetone
KCN	50 μ M	15
	500 μ M	8
	5 mM	—
CoA	1.7 mM	73
L-Penicillamine	0.4 mM	67
	2.4 mM	30
		27
<i>p</i> -Chloromercuribenzoate	44 μ M	61
	88 μ M	28
Iodoacetamide		
	10 mM	69
FeSO ₄	100 μ M	83
	1 mM	69
	5 mM	35
Haemin	10 μ M	101
	100 μ M	80
	600 μ M	29

in this way, especially if stable enzyme-inhibitor complexes were formed. The need for a higher concentration of glycine to increase the production of aminoacetone to that of aminolaevulate (Fig. 2) could be explained similarly. Also, it might be expected that aminolaevulate formation, being more dependent on pyridoxal phosphate than is aminoacetone formation, would be more inhibited by Fe²⁺ ions (Table 8), which are chelated by pyridoxal phosphate (Burnham & Lascelles, 1963). Again, the greater stability of the synthetase catalysing aminoacetone formation in preincubation experiments is consistent with its having a stronger enzyme-pyridoxal bond than that concerned with aminolaevulate formation. It is also possible that the decrease in the rate of aminolaevulate formation relative to the rate of aminoacetone formation produced by raising the temperature of incubation from 37.5° to 47° is related to a weaker enzyme-cofactor bond in aminolaevulate synthesis. Finally, variations in the standard method of preparation of particles (Table 4) and of storage of particles in different buffers or at a different pH (Table 6) all affected the production of each amino ketone differently, and these observations support the existence of separate synthetases (or isoenzymes).

Metabolic significance of aminoacetone. The potential importance of aminoacetone was first

suggested by Shemin (1955). Numerous functions have been proposed for aminoacetone, including participation in a metabolic cycle (Elliott, 1959; Green & Elliott, 1964), acting as a precursor to the D-1-aminopropan-2-ol part of vitamin B₁₂ (Neuberger & Tait, 1960; Turner, 1966, 1967) and taking part in the control of porphyrin metabolism (Neuberger & Tait, 1962). Whatever function aminoacetone may have in erythrocytes it is not utilized when incubated with freeze-dried particles obtained from avian erythrocytes (Gibson *et al.* 1958), with whole or lysed guinea pig erythrocytes (Urata & Granick, 1963), with rat blood (Green & Elliott, 1964) or with fresh particles or lysates of blood obtained from the domestic hen (R. A. Dale, unpublished work).

In accounting for a possible function of aminoacetone in avian erythrocytes three points must be considered. First, aminoacetone is formed in amounts up to 40% of aminolaevulate *in vitro*; secondly, the synthetase concerned with aminoacetone formation differs in a number of properties from aminolaevulate synthetase; thirdly, the production of both amino ketones is increased in anaemia produced by phenylhydrazine. These three features of aminoacetone formation would be satisfactorily accounted for if aminoacetone as well as aminolaevulate were involved in the formation of haemoglobin. Thus it is possible that the turnover of a metabolic cycle of the kind suggested by Elliott (1959) would increase during protein synthesis and this would involve an increase in the activity or amount, or both, of the enzymes, concerned. However, even if aminoacetone is produced in erythrocytes *in vivo* it could be utilized elsewhere. Thus Elliott (1960) showed that aminoacetone is oxidized to methylglyoxal by ox plasma.

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