Metabolism of 5-Nitro-2-furaldehyde Acetylhydrazone-C^{14} (Nihydrazone) in the Chick

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The accumulation and depletion of carbon-14 in tissues have been studied in the chick with formyl- and acetyl-C^{14}-nihydrazone. Tissue equilibrium of carbon-14 was established within 4 days. A biphasic disappearance curve was observed with liver and kidney following withdrawal of C^{14}-nihydrazone medication. The presence of isotopic residues after a long withdrawal period and slow turnover rates indicated labeling of normal body constituents. Saturation of these pools required 8 to 16 days of medication. A balance study with formyl-C^{14}-nihydrazone showed the following distribution of carbon-14: $C^{14}O_2$, 2.52%; droppings, 100.45%; and retention, 2.15%. Tissues from a distribution study when examined for labeled natural products showed the following C^{14}-labeled natural materials: glutamic acid, glycine, cystine, serine, aspartic acid, tyrosine, liver glycogen, and several blood proteins. These results are in accordance with a proposed hypothesis for the direct transformation of nihydrazone to natural products via α-ketoglutaric acid.

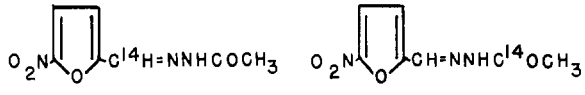
The development of nihydrazone (5-nitro-2-furaldehyde acetylhydrazone) as a feed additive in the treatment and control of various poultry diseases (*1*, *2*, *19*, *24*) resulted in a number of studies concerning the metabolic fate of this compound in the chicken. Several urinary metabolites, including 5-amino-2-furaldehyde acetylhydrazone and 5-acetamido-2-furaldehyde acetylhydrazone, have been identified following oral administration of nihydrazone to rabbits (*18*). These metabolites were no longer detectable 24 hours following oral administration of a single dose (*17*).

In the chicken, failure to detect nihydrazone at levels above 0.1 p.p.m. in tissues assayed (liver, kidney, muscle, fat, and skin) following a 15-week feeding period (no withdrawal) showed that nihydrazone does not accumulate in tissues during chronic administration (*3*). These results did not preclude the possible accumulation of nihydrazone metabolites in the tissues.

A study therefore was conducted to determine carbon-14 accumulation in the tissue of the chick during chronic administration of C^{14}-labeled nihydrazone and depletion of radioactivity following withdrawal of medication. Chemical studies have suggested that α-ketoglutarate might be formed directly from the 5-nitro-2-furaldehyde moiety of nihydrazone (*12*). The studies reported were initiated to determine whether nihydrazone-C^{14} is converted to labeled normal body constituents in the chick. Observed radioactivity in normal metabolites, particularly glutamic acid, following administration of radioactive nihydrazone is reported and a mechanism for its formation is suggested.

Methods

Administration of Drug. For the short-term accumulation and depletion and long-term accumulation experiments, the birds were housed in wire-floored cages and feed and water were provided *ad libitum*. Medicated feed, containing the appropriate radioactive compound, was prepared by diluting radiochemically pure (established by paper chromatography) C^{14}-nihydrazone labeled in the formyl or in the acetyl group with unlabeled nihydrazone to give specific activities desired, and blending the drug with a starter chick diet to give a feed containing 0.0138% nihydrazone.



A

B

A. Formyl-C^{14}-nihydrazone *B*. Acetyl-C^{14}-nihydrazone

For the short-term accumulation studies, sexed 1-week old White Leghorn chicks were prefed a starter ration containing 0.0138% nonradioactive nihydrazone for 3 weeks. Radioactive medicated feed was then fed *ad libitum* for 4 days. On the fourth day, the radioactive feed was removed and unmedicated feed was administered. At designated time intervals during the medication and withdrawal, randomly selected chicks were sacrificed and tissues analyzed for radioactivity.

The experimental procedure used for determining saturation of slow turnover pools involved administering radioactive feed for a maximum period of 32 days to week-old chicks not previously prefed nihydrazone. Chicks were removed from radioactive feed at designated times, fed an unmedicated diet for 4 days to allow the rapidly excreted metabolites to be depleted, and sacrificed, and tissue carbon-14 levels determined.

In the distribution study, 2-week old White Leghorn chicks were prefed unlabeled nihydrazone for 5 weeks. Three days prior to administration of radioactive nihydrazone, one male chick was placed in a large glass metabolism unit. After a 3-day acclimation period on nonradioactive medicated feed, formyl-C^{14}-nihydrazone (30.9 μc. per mg.) medicated feed was offered *ad libitum* to the chick. The chick was fed this diet for 8 days, then an unmedicated feed for 4 days, and was sacrificed. Excretion of radioactivity as respiratory CO_2 and excreta was determined daily during the 12-day period. Total respired CO_2 was trapped in 5*M* NaOH and determined by titration with standard HCl. For carbon-14 analysis, 0.5 ml. of the aqueous alkali solution was suspended in the thixotropic gel of Gordon and Wolfe (*8*) and counted. Droppings were collected in 3 liters of

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0.5*M* NaOH in the bottom chamber of the metabolism unit. Each collection was dissolved by heating and adding alkali as necessary, and the volume brought back to 3 liters with water. One milliliter of this solution was diluted to 10 ml. and a 0.5-ml. aliquot was added to the gel for carbon-14 assay.

Preparation of Tissue Samples for C^{14} Assay. The chicks were decapitated and blood was collected in heparinized tubes. The tissues were rapidly excised, frozen in dry ice, and stored in the freezer at −4° C. until required for assay. In the accumulation and depletion studies, the following tissues were assayed for carbon-14 concentration: liver, kidney, adipose fat, breast, leg, and thigh muscles. All the tissues and blood were assayed for radioactivity in the distribution study (see Table V). Carbon-14 residue in tissue was tabulated as nihydrazone equivalents based on the assumption that the average molecular weights were of the same order as that of nihydrazone.

Two grams of liver or 4 grams of all the other tissues, except fat, were homogenized in 10 ml. of absolute methanol using a Potter-Elvehjem homogenizer. One milliliter of the homogenate was suspended in the thixotropic gel (*8*) and counted in the liquid scintillation spectrometer. A maximum of 400 mg. of fat (depending on amount of sample available) was placed in a counting vial containing 3 ml. of toluene and allowed to stand at room temperature overnight. Thixotropic gel was added; the contents were mixed thoroughly and counted. For the distribution study the same procedure for tissue carbon-14 assay was followed except that the tissues were weighed before freezing and a smaller sample size was used. After all the tissues were excised, the remaining carcass was ground in a meat grinder and stored frozen. For carbon-14 assay, the ground carcass was dissolved in 2 liters of 5% KOH. Several days at boiling temperature and agitation were required for solution. After the volume was adjusted to 2 liters, a 25-ml. aliquot was diluted to 50 ml., and 0.5 ml. of the latter dilution was suspended in the gel. Two-tenths milliliter of whole blood was suspended in the gel immediately after collection.

All samples were counted in the Packard liquid scintillation spectrometer. Optimal counting conditions were obtained at a photomultiplier voltage of 1050 volts with a 10- to 100-volt window. Samples were corrected for background and counted until enough counts accumulated to give a standard deviation of 5% or less. All samples were corrected for quenching by the addition of internal standard. Efficiencies ranged from 10% for kidney and blood to 50 to 60% for muscle and fat.

Isolation of C^{14}-Labeled Metabolites. The tissues and biological fluids used in isolating specific metabolites were taken from the chick administered formyl-C^{14}-nihydrazone in the distribution experiment. Glutathione was isolated as the cadmium salt using the method of Goldzieher, Besch, and Velez (*7*) and analyzed colorimetrically using the procedure described by Flanagan *et al.* (*6*). The amino acid components of acid-hydrolyzed glutathione were separated by ion exchange chromatography (*10*). Those fractions that contained the same amino acid were pooled, evaporated to dryness, and taken up in 1 ml. of water. An aliquot was suspended in gel for carbon-14 assay. Verification of the radiopurities of the isolated amino acids was obtained by chromatography and liquid scintillation counting of the chromatogram (*13*). The amino acids of hydrolyzed serum proteins were separated by the method of Moore and Stein (*14*). Fractions containing the same amino acid were combined, concentrated, and counted in the liquid scintillation spectrometer.

Liver glycogen was isolated from fresh chilled liver (*9*, *20*) and hydrolyzed. The resulting glucose was purified by column chromatography (*23*), counted, and determined quantitatively with anthrone (*20*).

For determining the distribution of radioactivity in blood, 2 ml. of heparinized blood was centrifuged to separate plasma from erythrocytes. The erythrocytes were washed twice with 1-ml. portions of 0.9% NaCl. For radioassay of each fraction (including washings), 0.2 ml. was suspended in thixotropic gel.

The distribution of radioactivity in various serum protein fractions was studied by paper electrophoresis (*11*). Serum was dialyzed against barbital buffer, pH 8.6, μ 0.05 for 24 hours prior to electrophoretic separation in a Reco Model E-800-2 electrophoresis apparatus. Stained and unstained electrophoretograms were cut into $^3/_4$-inch segments and each segment was counted in the liquid scintillation spectrometer (*13*).

The hemoglobin from lysed erythrocytes was separated by electrophoresis using the technique of Motulsky, Paul, and Durrum (*15*), or alternatively by gel filtration with Sephadex G-25 with 0.9% NaCl as eluent.

Results and Discussion

Table I shows that equilibrium of carbon-14 in most of the tissues is established within 4 days on formyl- and acetyl-C^{14}-nihydrazone. The only exceptions to this general observation were kidney and fat in the acetyl-C^{14}-nihydrazone experiment. These differences were expected, since it was observed previously that 61% of the radioactive dose of acetyl-C^{14}-nihydrazone was expired as $C^{14}O_2$ (*16*), indicating extensive deacetylation of the acetylhydrazone moiety. Acetate can be a biochemical precursor of fatty acids through conversion to acetyl-coenzyme A, accounting for accumulation of radioactivity in the fat depots. Several investigators have reported that kidney is a high acetate metabolizing organ (*5*, *22*). This may account for the high levels of carbon-14 residues found in the kidney.

The depletion of radioactivity following the withdrawal of drug is shown in Tables II and III. Demonstrable levels of C^{14} residues were found after withdrawal periods as long as 16 days. Loss of C^{14} activity in kidney and liver following withdrawal of the drug (Figures 1 and 2) illustrates biphasic disappearance curves. These data indicate that sequential metabolic processes having different rates were in operation. The rapidly disappearing metabolites had half-lives ranging from 0.6 to 1.1 days (kidney and liver). The broken lines in Figures 1 and 2 represent the disappearance of

Table I. Concentration of Radioactivity in Tissues of Chicks Fed Radioactive Nihydrazone, P.P.M.

		Days on Medication					
		Formyl-C^{14}-nihydrazone			Acetyl-C^{14}-nihydrazone		
Tissue	Sex	1[a]	3	4	1	3	4
Liver	M	5.30[b]	8.13	6.30	7.78	15.6	10.2
	F	5.90	8.03	6.60	8.12	12.3	13.2
Kidney	M	7.95	5.80	7.10	8.84	20.0	35.0
	F	6.51	6.69	7.20	11.10	24.2	30.5
Thigh muscle	M	...	2.47	2.34	2.06	6.29	6.80
	F	1.55	2.53	1.88	2.17	3.44	3.59
Leg muscle	M	1.61	2.60	2.00	2.05	4.02	2.95
	F	...	2.95	1.28	1.67	3.85	3.19
Breast muscle	M	1.16	2.82	1.63	1.52	3.01	3.25
	F	1.11	2.22	1.66	1.74	3.61	2.99
Fat	M	1.33	...	...	9.84	58.5	91.3
	F	1.38	...	...	21.80	34.1	57.5

[a] Parts per million calculated as nihydrazone equivalents.
[b] Values for all the tissues except fat are averages of duplicate samples.
Specific activities of C^{14}-nihydrazones in medicated feed: Formyl-C^{14}, 3.75 μc./mg. Acetyl-C^{14}, 4.4 μc./mg.

Table II. Depletion of C^{14} from Tissues of Chicks Fed Formyl-C^{14}-nihydrazone, P.P.M.

		Days after Withdrawal of Medication				
Tissue	Sex	0[a]	1½	3	4	5
Liver	M	6.30[b]	3.53	2.16	1.54	1.36
	F	6.60	3.22	2.14	1.88	1.64
Kidney	M	7.10	4.17	2.11	1.49	1.27
	F	7.20	4.49	2.27	1.89	1.64
Thigh muscle	M	2.34	2.75	1.27	0.87	0.80
	F	1.88	1.58	1.05	0.97	0.96
Leg muscle	M	2.00	1.91	1.22	0.79	0.85
	F	1.28	1.61	1.21	1.13	0.96
Breast muscle	M	1.63	1.57	1.19	0.94	0.79
	F	1.66	1.56	1.07	0.97	0.95
Fat	M	...	0.95	0.75	0.65	0.86
	F	...	0.52	0.46	0.79	0.70

[a] Parts per million calculated as nihydrazone equivalents. Values for all tissues except fat are averages of duplicate samples.

Table III. Depletion of C^{14} from Tissues of Chicks Fed Acetyl-C^{14}-nihydrazone, P.P.M.

		Days after Withdrawal of Medication							
Tissue	Sex	0[a]	1½	3	5	8	12	14	16
Liver	M	10.2[b]	5.51	3.49	1.83	1.35	...	0.62	...
	F	13.2	5.45	3.75	2.72	...	0.77	...	0.54
Kidney	M	35.0	26.5	10.2	4.75	3.33	...	2.12	...
	F	30.5	29.0	10.4	6.84	...	2.46	...	1.28
Thigh muscle	M	6.80	2.73	3.63	2.26	1.29	...	0.76	...
	F	3.59	2.42	3.15	2.30	...	1.27	...	0.99
Leg muscle	M	2.95	2.95	2.14	1.22	1.17	...	0.85	...
	F	3.19	2.54	2.27	1.47	...	0.92	...	0.75
Breast muscle	M	3.25	2.58	1.72	1.41	1.18	...	0.96	...
	F	2.99	2.11	2.09	1.43	...	0.76	...	0.70
Fat	M	91.3	24.1	25.1	21.5	20.30	...	2.46	...
	F	57.5	37.7	38.0	26.5	...	13.4	...	4.4

[a] Parts per million calculated as nihydrazone equivalents. [b] Values for all tissues except fat are averages of duplicate samples.

metabolites having fast turnover rate. This was determined by subtracting the slow turnover rate from the portion of the curve having the greater slope. The slowly disappearing carbon-14 residues had half-lives ranging from 3 to 5 days.

The biphasic disappearance of carbon-14 residues observed in various tissues after cessation of medication with C^{14}-nihydrazone and the existence of residues after a long withdrawal period may indicate the presence of radioactive natural products. The possible occurrence

of labeled normal body constituents led to a long-term study of C^{14} saturation in the normal body constituents. Table IV shows that the carbon-14 content of body tissues is saturated within 8 to 16 days of medication with labeled nihydrazone. Under the conditions of this experiment (4-day withdrawal period), the contribution to the residual radioactivity by the rapidly excreted metabolites is negligible. Based on the half-lives of the rapidly excreted metabolites ($T_{1/2}$-1 day), a 4-day withdrawal period would result in a 10-fold decrease (90%) in the tissue concentration of these drug metabolites.

In a tissue distribution experiment with formyl-C^{14}-nihydrazone, all tissues contained appreciable radioactivity 4 days after withdrawal of radioactive feed (Table V). The range of radioactivity varied less than 10-fold, indicating relatively uniform distribution of labeling throughout the animal. Tissue retention accounted for 2.17% of the total dose 4 days after cessation of medication. The daily rate of C^{14} excretion in expired $C^{14}O_2$ and droppings is presented in Figures 3 and 4, respectively. Of the total dose administered, 2.52% was expired as $C^{14}O_2$ and 100.45% was excreted in the droppings; 105.14% of the administered dose was recovered. The distribution of radioactivity in blood showed the following: plasma 14.8%, washed erythrocytes 74%, and washings 13.8%.

The rapid disappearance phase of carbon-14 depletion (Figures 1 and 2) may be correlated with the excretion of metabolites such as 5-amino- and 5-acetamido-2-furaldehyde acetylhydrazones (*18*). The disappearance of these and other urinary metabolites from the chicken after oral administration of formyl-C^{14}-nihydrazone proceeds with a biological half-life of 3 hours (*17*).

The mechanism by which normal body materials could incorporate the radioactive tracer from nihydrazone was suggested by other studies (*12*). Certain nitrofuran compounds are reduced both chemically (*4*) and biologically (*18*) to the corresponding aminofurans.

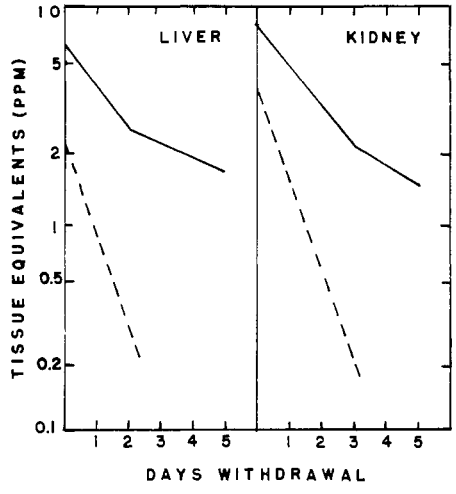

Figure 1. Tissue depletion of radioactivity from chicks fed formyl-C^{14}-nihydrazone

$T_{1/2}$ of slow turnover rate in days: Liver, 3.2; kidney, 3.4.
$T_{1/2}$ of fast turnover rate in days (broken line): Liver, 0.6; kidney, 0.7

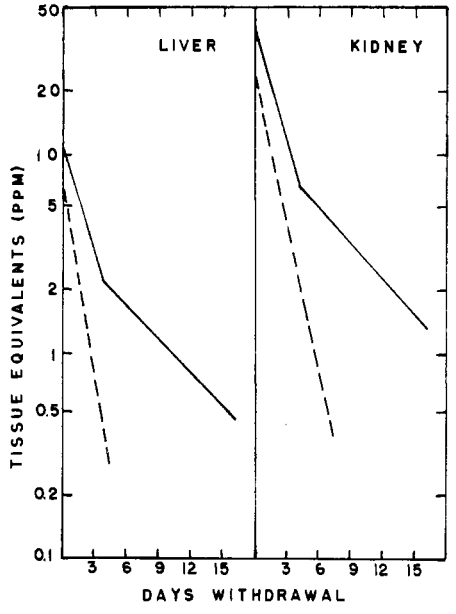

Figure 2. Tissue depletion of radioactivity from chicks fed acetyl-C^{14}-nihydrazone

$T_{1/2}$ of slow turnover rate in days: Liver, 5.2; kidney, 5.4
$T_{1/2}$ of fast turnover rate in days (broken line): Liver, 1.0; kidney, 1.0

Table IV. Long-Term Tissue Accumulation of Formyl- and Acetyl-C^{14}-nihydrazone Residues in the Chick, P.P.M.

	Days on Medication[a]							
	Formyl-C^{14}-nihydrazone				Acetyl-C^{14}-nihydrazone			
Tissue	4[b]	8	16	32	4	8	16	32
Liver	1.54[c]	3.22	3.35	3.99	2.73	4.15	5.82	6.02
Kidney	1.49	2.50	3.35	3.58	14.09	18.30	18.40	14.02
Thigh muscle	0.87	1.47	1.64	2.18	3.28	6.86	6.20	9.36
Leg muscle	0.79	1.35	1.78	2.17	3.32	7.59	5.39	6.23
Breast muscle	0.94	1.31	1.91	2.05	3.70	5.15	5.44	5.96
Fat	0.65	0.64	0.26	0.95	54.70	88.50	70.50	58.70

[a] Each feeding period was followed by a 4-day withdrawal of drug before sacrifice.
[b] Parts per million calculated as nihydrazone equivalents.
[c] Values for all the tissues except fat are averages of duplicate samples.
Specific activities of nihydrazone in medicated feed: Formyl-C^{14}, 0.625 μc./mg. Acetyl-C^{14} 0368, μc./mg.

Table V. Distribution of C^{14} in the Chick 4 Days after Withdrawal of Formyl-C^{14}-nihydrazone

Tissue	P.P.M.[a]
Liver	4.00[b]
Kidney	4.18
Breast muscle	1.86
Leg muscle	1.37
Thigh muscle	1.98
Fat	1.38
Heart	1.90
Brain	2.57
Lung	2.36
Spleen	2.04
Intestine and contents	0.59
Gall bladder	1.70
Proventriculus and contents	1.83
Ceca and contents	2.21
Pancreas	1.55
Testis	1.26
Crop and contents	0.51
Skin	1.39
Gizzard	2.64
Gizzard contents	0.19
Gizzard lining	1.20
Blood	1.38
Carcass	1.77

[a] Parts per million calculated as nihydrazone equivalents.
[b] Values for all tissues, organs, fluids, and contents are averages of duplicate samples. Specific activity of formyl-C^{14}-nihydrazone in medicated feed, 30.9 μc./mg.

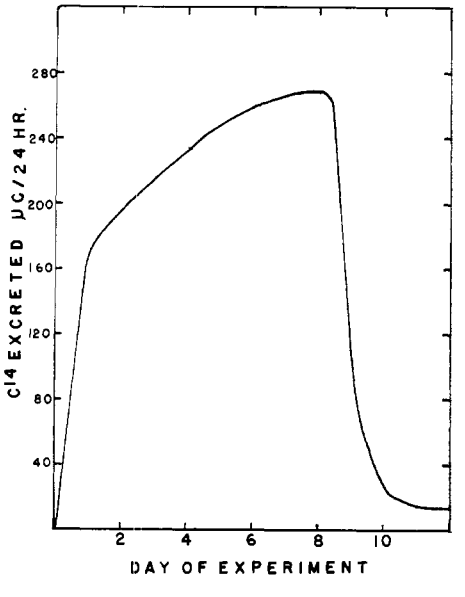

Figure 3. Expiration of $C^{14}O_2$ by the chick fed formyl-C^{14}-nihydrazone

Radioactive feed withdrawn on eighth day of experiment

Chemical evidence for the mechanism of the conversion of aminofurans to α-ketoglutaric acid was obtained by subjecting ethyl-5-amino-2-furoate to alkaline hydrolysis; α-ketoglutaric acid was isolated as a dinitrophenylhydrazone derivative from the reaction (*12*).

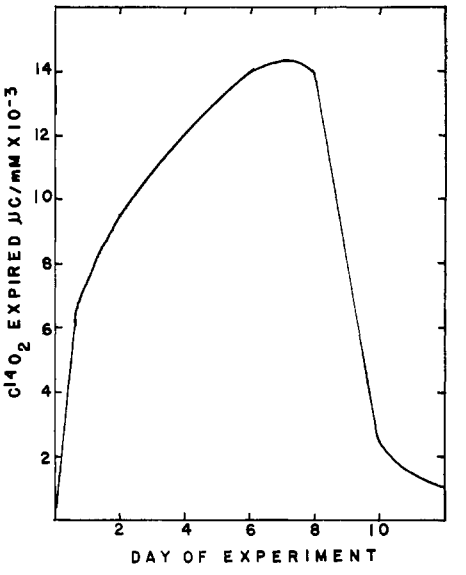

Figure 4. Urinary and fecal excretion of C^{14} by the chick fed formyl-C^{14}-nihydrazone

Radioactive feed withdrawn on eighth day of experiment

Glutamic acid, a readily available metabolite of α-ketoglutaric acid, was isolated in order to provide direct evidence for the conversion of C^{14}-nihydrazone to C^{14}-labeled normal body constituents. Glutathione, a peptide containing glutamic acid, was used as a source for the identification of labeled glutamic acid. From approximately 1 ml. of erythrocytes, 1.4 mg. of glutathione was isolated, the specific activity of which was 10,466 d.p.m. per mg. Acid hydrolysis of the erythrocyte glutathione and subsequent isolation of glutamic acid demonstrated that this amino acid was labeled. Radiochemical purity of isolated glutamic acid was verified by paper chromatography. Radioactive glycine and cystine also were isolated from hydrolyzed liver glutathione and subsequently verified as radiochemically pure by paper chromatography.

Aspartic acid, serine, glutamic acid, and tyrosine separated from erythrocyte protein hydrolyzates also were significantly radioactive. Proline, methionine, lysine, and arginine were separated and nonradioactive. The other amino acids were not analyzed.

Glucose obtained from liver glycogen had a specific activity of 110 d.p.m. per mg. of glycogen. Radioactivity was detected in the albumin fraction of chick serum and only traces of activity in the globulin fractions. The inability to demonstrate appreciable radioactivity in the globulin fraction is considered to be due to lower concentrations of globulin in chicken serum relative to albumin (*21*). The radioactivity of hemoglobin was demonstrated in the electrophoretogram and as the isolated protein.

The observation of radioactivity in glutamic acid and other normal body constituents demonstrated the incorporation of carbon-14 from nihydrazone into normal metabolites in the chick. Figure 5 depicts a reasonable

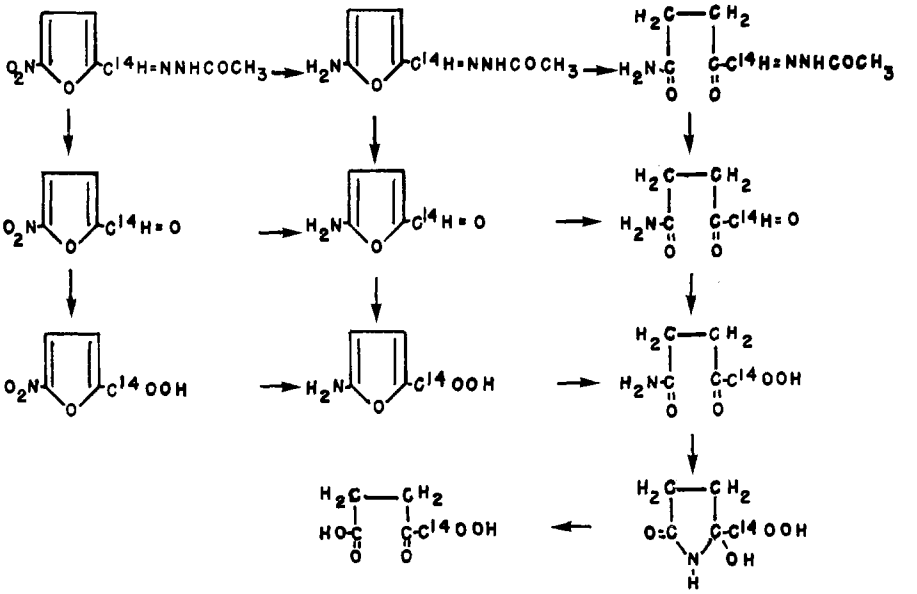

Figure 5. Possible mechanisms for the conversion of formyl-C^{14}-nihydrazone to a α-ketoglutaric acid

mechanism leading to the formation of α-ketoglutaric acid from nihydrazone. The presence of aminofurans as metabolites of nitrofurans and the finding of radioactivity in normal metabolites support this theory. The data given here are in accordance with the hypothesis that nitrofurans, containing the furaldehyde moiety coupled with an azomethine linkage, can be metabolized directly to naturally occurring materials via α-ketoglutaric acid. These results do not rule out other pathways for carbon-14 incorporation into natural products. The higher levels of tissue carbon-14 residues with acetyl-C^{14} suggests that $C^{14}O_2$ fixation might occur to some extent in the chicken fed C^{14}-nihydrazone. With acetyl-C^{14}-5-nitro-2-furaldehyde acetylhydrazone there was a 20-fold increase in $C^{14}O_2$ expiration over that observed with the formyl-C^{14} nitrofuran. Residues with acetyl-C^{14} were generally twice the level observed for formyl-labeled compound. The reason for high fat and kidney C^{14} levels has been mentioned. This comparison indicates the relatively minor importance of CO_2 fixation in the incorporation of C^{14} from formyl-C^{14}-nihydrazone into natural products.

Acknowledgment

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