Table II. Total Arsenic Retention (μg. per Rat)

	Dietary Arsenic Source					
	Natural		As-Liver		As_2O_3	
	M	F	M	F	M	F
Total intake	65	60	4700	4350	4500	4200
Carcass[a]	6.2	5.5	246	243	317	270
Liver	0.54	0.47	72	76	105	98
Total body[b]	11.8	10.5	519	518	681	589
Retention (%)	18.3	17.7	11.1	11.8	15.1	14.5

[a] Entire animal minus abdominal viscera and skin.
[b] Calculated values based on carcass = 55% of live weight, and assuming uniform distribution.

Table III. Arsenic Balance Data[a] (4th Week)

	Dietary Arsenic Source					
	Natural		As-Liver		As_2O_3	
	M	F	M	F	M	F
Diet level (p.p.m.)	0.22	0.22	16	16	16	16
Food intake (grams)	87	75	84	75	88	74
As intake (μg.)	19	17	1340	1200	1410	1185
As excretion						
Feces (μg.)	1.2	1.5	284	233	128	96
Urine (μg.)	1.5	2.6	202	260	327	170
U/F	1.2	1.8	0.71	1.1	2.5	1.8
Total excreted (%)	14	24	36	41	32	22

[a] Five rats of each sex per group.

The small number of animals used did not warrant statistical treatment of the data, and the average data served adequately to demonstrate the effects reported.

Discussion

The ability of the liver to store arsenic from dietary sources, especially at high levels of intake, has been repeatedly demonstrated. The use of the turkey, in the present instance, was expedient in obtaining a convenient source of protein-bound arsenic which, on the basis of Winkler's work (*3*), would be expected to exist almost entirely in the pentavalent organic state. The fact that rats stored arsenic from this source with nearly as much facility as they did from a trivalent inorganic source is not in agreement with the observations of Overby and Frost on pork liver from swine fed arsanilic acid. It seems unlikely that either the difference in species (turkeys versus swine) or the different arsenical compound fed (*p*-ureidobenzenearsonic acid versus arsanilic acid) would be entirely responsible for the divergent results in the two studies. It appears reasonable to suppose however that rats still in the rapid phase of growth, as were those used in this study, may deposit arsenic in their tissues more readily, from either type of dietary source, than would more mature animals. Furthermore, the dietary arsenic level in the present work was more than twice that in the Overby and Frost study, besides which the total arsenic intakes were six to seven times as great.

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Received for review October 11, 1962. Accepted June 3, 1963. Division of Agricultural and Food Chemistry, 142nd Meeting, ACS, Atlantic City, N. J., September 1962.

FEED ADDITIVES

Metabolic Stability of Radioactive Arsanilic Acid in Chickens

L. R. OVERBY[1] and R. L. FREDRICKSON

Biochemistry Research Department, Research Division, Abbott Laboratories, North Chicago, Ill.

THE AGE of chemotherapy actually began in the early 1900's with the use of organic arsenicals as parasitological agents. Arsanilic acid was the first "magic bullet" of Paul Ehrlich. The mechanism of its action is still unknown, despite the long history of use, first as a parasiticide, and, more recently, as an ingredient in medicated animal feeds.

The effect of arsanilic acid in chemotherapy was a paradox. It was inactive against parasites in vitro; and in vivo required a latent period, during which there was no parasiticidal action. There were several attractive hypotheses to explain this enigma. Ehrlich (*2*) believed there must be reduction to the arsenoxide to account for the delayed in vivo activity. Others suggested the possibility of a slow release of inorganic arsenic. Igersheimer (*3*), in 1909, reported some inorganic arsenic was excreted after arsanilic acid injection in rabbits. Nierenstein (*4*), also in 1909, reported a similar finding in horses. However, the most striking fact about all the early German literature is that in almost all experiments, arsanilic acid was excreted completely unchanged. There was little support for Ehrlich's reduction theory.

There were other hypotheses to account for the action of arsanilic acid—for example, an active principle composed of an arsenical-protein complex, or increased antibody-forming systems under the influence of arsanilic acid. But this was in 1909, and the experiments possible then, although elegant for the time, were crude by modern biochemical techniques.

The question of mechanism still remains unanswered, but the idea that organic arsenicals are degraded metabolically to inorganic arsenic, or reduced to the arsenoxide, has persisted, mainly because of reiteration, and not because of verifying research. The early circumstantial evidence is often sanctified by repeated quotation.

Long-term experience with arsanilic acid in this laboratory, coupled with a critical evaluation of available literature, indicated that if this arsenical were metabolically altered at all, it was in an extremely small amount. It is axiomatic that only the positive can be proved with certainty. To prove a universal

[1] Present address: Department of Microbiology, University of Illinois, Urbana, Ill.

Arsanilic acid appeared metabolically stable as used in medicated feeds of chickens. The ratio of C^{14} to As^{74} in a double-labeled molecule did not change. Unlike arsenate-As^{74}, no radioactivity was expired from arsanilic-As^{74} acid. Arsanilic acid was found unchanged in excreta. Neither arsenate metabolic products nor reduced forms of arsanilic acid were found in tissues. Residual arsanilic acid in edible tissues was handled like authentic arsanilic acid in second animals.

negative, the absolute absence of metabolic degradation, of course, is impossible. As quantitative methods are refined, the goal of zero only moves farther away.

The possible metabolic alterations of arsanilic acid are shown in Figure 1. Research thus far indicates that none of these things happens to arsanilic acid when it is consumed orally by chickens. This conclusion is based on experimental work done with As^{74}- and C^{14}-labeled tracers. Details will be presented in later publications, but essential points for the present discussion follow. First, in a double-labeled, C^{14}-, As^{74}-arsanilic acid, the two isotopes remained in constant proportions in all the various body compartments. Second, there was no expired As^{74} after administration of arsanilic-As^{74} acid. Third, arsanilic acid was excreted intact. Fourth, arsenate metabolites were not detected in tissues of chickens given arsanilic acid. Finally, tissues containing arsanilic acid residues were handled like authentic arsanilic acid when fed to second animals (*5*, *6*), including man (*7*).

A rupture of the carbon-arsenic bond in arsanilic-1-C^{14}, -As^{74} acid would leave C^{14}-labeled aniline and As^{74}-labeled arsenate. Through normal differential metabolism the two isotopes would go their separate ways. They would not stay together in any consistent manner. But if the bond were not ruptured, the ratio of C^{14} to As^{74} would always remain constant, regardless of whatever happened to the rest of the molecule. Chicks were fed double-labeled arsanilic acid synthesized in this laboratory, and then counting rates of the two isotopes were differentially determined in tissues taken 48 hours after the dose. The following body compartments were sampled: blood, muscle, heart, lung, spleen, feathers, gizzard, bone, intestinal wall, liver, kidney, bile, intestinal contents, and excreta. The results of six such experiments are condensed and shown in Table I. The first two columns show that the double-labeling technique works. Chick 1 received a dose composed of 99.95% of arsanilic-1-C^{14} acid plus 0.05% of inorganic arsenate-As^{74}. This was to measure the differential retention and excretion of arsanilic acid with a trace of arsenate. The results showed that a massive amount of arsanilic acid was excreted faster than even a trace of arsenate. Chick 2 received the double-labeled arsanilic acid plus 1% of added

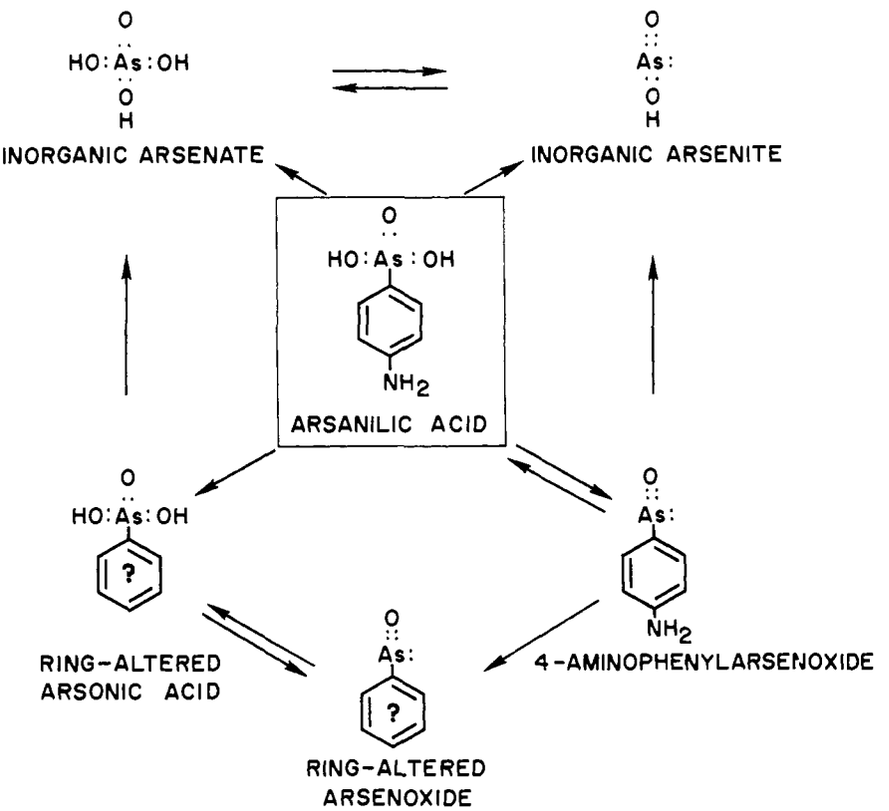

Figure 1. Pathways of possible metabolic alteration of arsanilic acid

Table I. Stability of C^{14}/As^{74} Ratios in Chicks Given Arsanilic-1-C^{14}, -As^{74} Acid

	Ratio of C^{14} to As^{74} for Chick No.					
	1	2	3	4	5	6
Dose[a]	6.3	0.9	2.3	1.4	0.9	1.8
Tissues[b]	2.1	0.6	2.2	1.3	1.0	1.7
Excretory[c]	10.7	1.2	2.3	1.4	0.9	1.8

[a] Dose = administered drug.
[b] Tissues = all body compartments, except those related to excretion.
[c] Excretory = liver, kidney, bile, intestinal contents, and excreta.

Table II. Excretion of Arsenic by Rats Fed Residual Arsanilic Acid and As_2O_3

	Arsenic Balance			Arsenic Recovery, %
	Ingested, μg.	Excreted, μg.		
		Feces	Urine	
Residual AA[a]	660	503	162	101
As_2O_3	668	210	151	51

[a] Liver protein from swine fed 0.05% of arsanilic acid in ration for 14 days.

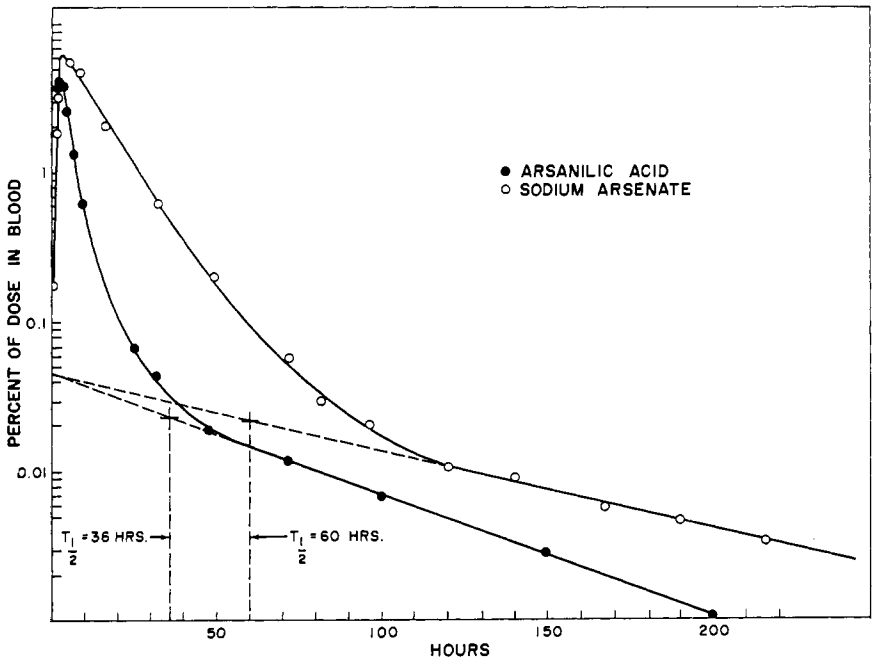

Figure 2. Rate of decrease of radioactivity in blood of chickens given a single oral dose of arsanilic acid-As^{74} followed by arsenate-As^{74}

arsenate-As^{74}. Here again, the isotope ratio decreased 30% in tissues and increased a similar amount in organs of excretion. Chicks 3, 4, 5, and 6 received the double-labeled arsanilic acid only. In each case, the dose ratio, tissue ratio, and excretory ratio of C^{14} to As^{74} were the same, within the limits of experimental error. Thus, if the carbon-arsenic bond of arsanilic acid were ruptured by chicks at all, it was much less than 1% of the administered dose. This was as close to zero as the double-label technique permitted. The limiting factor was proof of the radiopurity of the labeled arsanilic acid. A trace of arsenate-As^{74} as an impurity would be detected through biological amplification. Trace radioactive impurities in the dose could not be distinguished from those produced metabolically.

It was found that arsenate-As^{74} is expired by chickens. If arsanilic acid is converted to arsenate endogenously, then this arsenate should also be expired. Chicks were given either arsenate-As^{74} or arsanilic-As^{74} acid, and then placed in a metabolism chamber designed to collect expired air. The air was passed through the chamber, then through a vibrating reed electrometer, and finally through a trap to collect volatile arsenic compounds. After a dose of 1.7×10^8 c.p.m. of arsenate-As^{74}, 981,000 c.p.m. were collected in the arsenic trap (about 0.6% of the dose). Radioactivity was first detected in the electrometer at about 8 hours after the dose, a maximum was reached at 24 to 48 hours, followed by a slow decline. After an equal dose of arsanilic-As^{74} acid, counts were not above background in the total air expired during 5 days. No radioactivity was detected passing through the electrometer. The vibrating reed is the most sensitive instrument available for detecting radioisotopes. Had even 0.1% of the arsanilic acid been converted to arsenate, and then this expired like exogenously administered arsenate, there would have been 981 c.p.m. of radio-As^{74} expired.

The above studies did not detect inorganic arsenic or its metabolic products in tissues of chicks given arsanilic acid. The nature of the transport forms should then be considered. Arsenicals are rapidly cleared from the body, but the true biological half-life has not heretofore been defined. The biological half-lives of arsanilic-As^{74} acid and arsenate-As^{74} were compared in the blood of chickens. A chicl was given an oral dose of arsanilic-As^{74} acid, and the level of radioactivity was determined serially in blood samples for a period of 10 days. After another 20 days, the same chick was given arsenate-As^{74}, and blood radioactivity was determined serially as before. The procedure was repeated with other chicks with arsenate given first, followed by arsanilic acid. The decay curves are shown in Figure 2. This semilog plot shows that the disappearance of arsanilic acid or arsenate from blood fits the equation of three decreasing exponentials. The biological half-lives may be determined graphically from these plots. Extension of the final straight line back to zero time indicates that less than 0.1% of the dose was present in the blood in the form represented by this line. The biological half-life for arsanilic acid was 36 hours, and 60 hours for arsenate. Not shown in this figure are the half-lives of the two other first-order clearances. They were 6 hours and about 90 minutes, respectively. These results indicate that about 99.9% of the arsanilic acid in blood clears out with a half-life of 90 minutes or 6 hours; the remaining 0.1% clears with a half-life of about 36 hours. These are first-order reactions, with the rate depending on the concentration of arsenical. The picture with arsenate is very similar, with the half-lives about doubled. The results with another animal given first arsenate, then arsanilic acid, were almost identical to the previous experiment. The half-lives were 38 and 63 hours. Prior dosing with either arsenical did not affect the blood levels of a subsequent one. Other experiments showed that the radioactivity in tissues cleared in a first-order reaction, very much the same as blood. The results were consistent with the idea that arsanilic acid was "translocated" in the body—not "transformed."

Table III. Blood to Tissue Arsenic Ratios in Chicks after 14 Days of Continuous Feeding of Three Types of Arsenic

	Blood to Tissue Ratio of Arsenic Ration		
Tissue	Control	As_2O_3	Residual AA[a]
Muscle	3.9	2.7	7.0
Liver	2.6	3.1	0.8
Kidney	3.2	6.7	1.7

[a] Liver protein from swine fed 0.05% of arsanilic acid in ration for 14 days.

The nature of the transport forms of arsanilic acid in various body compartments was studied. Chicks were fed radioarsanilic acid for 5 days. Then after allowing 48 hours for clearance of the unchanged arsenical, the animal was sacrificed, and extracts of tissues and organs were made. More than 90% of the radioactivity could be extracted with a mixture of pyridine, formaldehyde, and water, described by Winkler (7). In this laboratory, several systems were developed for identifying arsenicals. The systems were: paper chromatography using acetonitrile-nitric acid-water (78:2:20, by volume), and isopropanol-water (70:30, by volume) developing systems; paper electrophoresis at varying pH; and ion exchange chromatography on Dowex 50 resin.

By using the above systems, two components, in addition to arsanilic acid, were characterized in extracts of tissues or excreta of arsanilic acid-fed chicks. Three components, in addition to a trace

of free arsenate, were characterized in arsenate-fed birds. None of the products of arsenate metabolism could be detected, even in trace amounts in arsanilic acid-fed chicks. *p*-Aminophenylarsenoxide, the reduced form of arsanilic acid, could not be detected in tissues or excreta. The tissue "bound-forms" of arsanilic acid appeared to be excreted via the liver and kidney, and then converted to free arsanilic acid in the intestine and excreta. The chromatographic and electrophoretic characteristics of the major bound arsanilic acid component suggested that it was a conjugate with a small acidic molecule via the arsenic. The other component was chemically very similar to arsanilic acid itself.

Tissue-bound arsanilic acid from swine liver or chick muscle has been fed to second animals, including rats (*5*), chickens (*6*), and man (*7*). The results are best characterized by saying that residual arsanilic acid in edible animal tissues is handled in second animals very much like authentic arsanilic acid.

In the study with rats, residual arsanilic acid was almost completely excreted via the liver and kidney during a 14-day feeding cycle. For comparison, rats excreted only about5 0% of As_2O_3 arsenic. Table II shows the average excretion of the two forms of arsenic. In another study, actual retention of residual arsanilic acid was studied in blood and tissues of chickens (*6*). The residual arsanilic acid in swine liver was partially absorbed by the chicks and appeared in blood and organs of excretion. However, as shown in Table III, the level of arsenic in muscle did not rise commensurate with the increased blood levels. On the other hand, the arsenic of As_2O_3 did appear in muscle and other tissues in proportion to the blood levels.

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Received for review October 11, 1962. Accepted August 6, 1963. Division of Agricultural and Food Chemistry, 142nd Meeting, ACS, Atlantic City, N. J., September 1962.

FEED ADDITIVES

A Carcinogenicity Evaluation of Potassium Arsenite and Arsanilic Acid

R. K. BOUTWELL

McArdle Memorial Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wis.

Tests were made to determine whether $KAsO_2$ might act as an incomplete carcinogen; thus it was tested in conjunction with agents able either to initiate (DMBA) or to promote (croton oil) skin tumors in mice. No activity for $KAsO_2$ was detected. Both benign and malignant skin tumors were induced in skin tumor-susceptible mice by means of a single application of DMBA followed by repetitive applications of croton oil. Both agents were used in amounts that did not produce maximal tumor yields. The influence of arsanilic acid and $KAsO_2$ on the yield of tumors was tested by including one of these arsenicals in the diet of the mice concurrently with exposure to DMBA or to croton oil. No change was found in the tumor incidence of the test mice attributable to the arsenicals.

Massive amounts of potassium arsenite ($KAsO_2$) have been reported to be carcinogenic for man with a special propensity to induce skin cancer (*9, 11*). In contrast, several investigators have concluded that there is no decisive evidence that exposure to arsenic compounds is causally related to ensuing cancer in man. Kenaway (*10*) rejected the evidence for arsenic carcinogenicity while Boyland (*3*) wrote ". . . no unequivocal evidence has ever been presented that arsenic is carcinogenic." More recently, Vallee *et al.* (*14*) stated that although a number of cases of cancer due to arsenic have been officially reported, "It is still questionable that a direct relationship was established." Frost (*6*) reached a similar conclusion.

With respect to the question of the experimental production of cancer by arsenicals, it is clear that no established method exists whereby one can produce tumors at will in laboratory animals (*5*). As the experiments with arsenic alone did not cause tumors, other ways were tried. Neubauer (*11*) has stated that Ciechanowski, Morozowa, and Wilhelmi (1925) observed an accelerating effect of oral ingestion of potassium arsenite on the development of tar carcinoma in rabbits, although both Schiller (1926) and Pucinelli (1930) reported a protective effect of arsenic on the appearance of tumors in the tarred skin of mice.

Further carcinogenicity evaluations of two arsenicals, $KAsO_2$ and arsanilic acid (AA), were undertaken using tests designed to reveal the smallest possible contribution of the test compound to tumor incidence. Because palmer and planter hyperkeratoses are sometimes observed in man after chronic systemic poisoning with arsenic (*7*), and in consideration of the hyperkeratotic lesions that develop on the skin of mice exposed to a single application to the skin of 7,12-dimethylbenz(a)anthracene (DMBA) followed by repetitive applications of croton oil (*1*), the role of $KAsO_2$ on the induction of skin tumors in mice by the two-stage process (*1, 2, 13*) was investigated. Even if the arsenicals were not complete carcinogens but