Technical Papers

Anomalous Thermal Behavior of Salivary Apoerythein Activity

Ernest Beerstecher, Jr., and Eleanor J. Edmonds

Department of Biochemistry, The School of Dentistry, The University of Texas, Houston

Eakin and co-workers have adapted a modified vitamin B_{12} assay to the measurement of apoerythein (pernicious anemia "intrinsic factor") activity (*1*) and demonstrated the presence of apoerythein in saliva (*2*). The assay depends upon the ability of apoerythein to bind vitamin B_{12}, making it unavailable for the growth of *Escherichia coli,* which depends upon the vitamin for growth in a specially defined medium. Apoerythein activity is thus indicated in the assay by inhibition of bacterial growth and is quantitated by comparison of the inhibition with that produced by a standardized solution of gastric mucosa. One unit of apoerythein is able to bind 1 μg of vitamin B_{12}, and the results are reproducible to within 5%. In studies on the thermal stability of salivary apoerythein we have now discovered that all activity disappears from saliva heated to 70° C for periods of 1 min or longer, but that samples heated for 5 min at either lower temperatures, or higher temperatures up to 100°, show no such loss in activity. Typical results are shown in Table 1. The data represent the

TABLE 1

EFFECT OF HEATING UPON THE ACTIVITY OF 1 MILLIUNIT SALIVARY APOERYTHEIN

Treatment temperature °C	Apparent activity of 1 milliunit apoerythein
30	1.00
60	.85
65	.45
70	0
75	.20
80	.80
100	1.00
90–70	.95
70–90	0

effects upon potency obtained by heating 1 milliunit of salivary apoerythein. All samples were heated for 5 min at the indicated temperatures except the last two, which were heated for 5 min at each of the indicated temperatures and in the order shown.

If samples are first heated to temperatures higher than 70° C and subsequently heated to 70°, there is no loss of activity. Samples heated at 70° C for longer than 1 min lose their activity and cannot be reactivated by subsequent heating to a higher temperature. It appears that the high rate of inactivation at 70° does not influence appreciably samples elevated through the critical temperature range at a sufficiently rapid rate. In our experiments less than 10 sec elapsed while the samples were being raised through the 65°–75° range. The peak inactivation temperature occurs at slightly over 70°, there being some inactivation at 75° and less at 65°, but no measurable amount at either 60° or 80°. It cannot be construed from these data that the over-70° salivary apoerythein activity has been unaffected by the thermal treatment, since it differs from normal salivary apoerythein activity in that it is immune to destruction at 70° for even prolonged periods.

References

1. TERNBERG, J. L., and EAKIN, R. E. *J. Am. Chem. Soc.,* **71,** 3858 (1949).
2. BEERSTECHER, E., JR., and ALTGELT, S. *J. Biol. Chem.,* **189,** 31 (1951).

Formation of α-Keto Acids from α-Amino Acids by the Action of Free Radicals in Aqueous Solution

G. R. A. Johnson, George Scholes, and Joseph Weiss

University of Durham, King's College, Newcastle upon Tyne, England

The mechanisms of metabolic processes are, in many instances, still a matter of some conjecture, and there has been some discussion (*1, 2*) in this respect, as to the possibility of the participation of free radicals. Thus the actual simulation of certain enzymatic processes by reactions involving free radicals *in vitro* may be important in any study of this nature.

It is now well established that the breakdown of α-amino acids by animal tissues consists of an oxidative deamination with the intermediate production of the α-keto acids (*3*). We have investigated, therefore, the action of OH and HO_2 radicals on a number of α-amino acids. These radicals have been produced in solution as follows:

(*a*) By the decomposition of hydrogen peroxide in the presence of a ferrous salt (Fenton's reagent), a reaction which proceeds as follows (*4*):

$$H_2O_2 + Fe^{2+} \longrightarrow OH + OH^- + Fe^{3+}. \qquad (1)$$

(*b*) By irradiation with x-rays of dilute aqueous solutions, where radicals are formed according to the net process (*5*):

$$H_2O \longrightarrow\!\!\!\!\!\!\wedge\wedge\wedge\longrightarrow H + OH, \qquad (2)$$

which in the presence of oxygen is followed by:

$$H + O_2 \longrightarrow HO_2. \qquad (3)$$

Previous studies on the oxidation of amino acids by Fenton's reagent (*6–8*) showed that these substances are deaminated and converted to the aldehydes and the corresponding carboxylic acids. However, with the exception of glycine, which affords glyoxylic acid (*6*),

the formation of the corresponding α-keto acids was not reported. Re-examination of the effects of this reagent on various amino acids (e.g., alanine, serine, and leucine) has now shown that α-keto acid formation does take place to some extent. A typical oxidation experiment was conducted as follows:

To a stirred solution of alanine (approx 0.01 moles) were added, dropwise and simultaneously, equimolecular amounts of ferrous sulfate and hydrogen peroxide. The reaction mixture, after acidification and addition of 2:4-dinitrophenylhydrazine solution, was allowed to stand a short while, and the hydrazones were then extracted with chloroform. This extract was washed and shaken with 10% sodium carbonate. The hydrazone of pyruvic acid was precipitated from the carbonate extract on addition of acid—mp, 217°–218° C (after recrystallization from ethanol); no depression of the mp on mixing with an authentic specimen; yield: ∽ 10 mg of pyruvic acid. Better yields may possibly be obtained by changing the experimental conditions.

There are only two other recorded cases of the chemical conversion of alanine to pyruvic acid. Simon and Piaux (*9*) used copper in the presence of oxygen, and Bass (*10*) employed ferrous bicarbonate, also in the presence of oxygen. It is very likely that these oxidations also involve free radicals.

Investigations of the effects of x-rays on aqueous solutions of α-amino acids have shown (*11, 12*) that, *in vacuo,* deamination and decarboxylation occur, with subsequent formation of the corresponding aldehyde (*11*). Some hydroxylamine is also produced under certain conditions (*13*). We have found that irradiation in the presence of oxygen results in the formation of both the α-keto acid and the aldehyde. From irradiated alanine solutions, for instance, pyruvic acid was isolated as the 2:4-dinitrophenylhydrazone. This was identified by its mp and mixed mp and was further characterized by paper chromatography in a butanol-water-NH_3 solvent (*14*).

Table 1 shows some quantitative data obtained at three selected pH values. The keto acid was determined as the 2:4-dinitrophenylhydrazone by the method of Friedemann and Haugen (*15*), and the ammonia by the distillation method of Parnas (*16*).

TABLE 1

IRRADIATION OF ALANINE IN AQUEOUS SOLUTION (0.2%) WITH X-RAYS (200 kv) IN THE PRESENCE OF OXYGEN (Dose = $3 \cdot 6 \times 10^4$ r-units)

pH	Yield in (μ moles/ml) × 10^{-2}	
	Pyruvic acid	Ammonia
2	4.70	7.53
7	4.67	5.73
9	4.50	11.0

It will be noticed that the yield of ammonia depends markedly upon pH, giving a minimum value in the region of the isoelectric point; this is in agreement with previous findings (*11*). Pyruvic acid formation, however, does not depend so strongly on pH.

Irradiation of alanine solutions *in vacuo* yields predominantly acetaldehyde (*11*). This suggests that the oxygen present in the irradiated solution plays a very important part in the mechanism of pyruvate production. It is thus highly probable that pyruvic acid and acetaldehyde are formed through two different intermediates. The mechanism of this reaction is now being studied in detail.

References

1. HABER, F., and WILLSTAETTER, R. *Ber.*, **64**, 2844 (1931).
2. STEIN, G., and WEISS, J. *Nature*, **166**, 1104 (1950).
3. KREBS, H. A. *Biochem. Soc. Symposium*, (1), 2 (1948).
4. HABER, F., and WEISS, J. *Proc. Roy. Soc.* (*London*), A, **147**, 332 (1934).
5. WEISS, J. *Nature*, **153**, 748 (1944); *Trans. Faraday Soc.*, **43**, 314 (1947).
6. DAKIN, H. D. *J. Biol. Chem.*, **1**, 171 (1905–6).
7. NEUBERG, C. *Biochem. Z.*, **20**, 531 (1909).
8. WIELAND, H., and FRANKE, W. *Ann.*, **457**, 1 (1927).
9. SIMON, L. J., and PIAUX, L. *Compt. rend.*, **176**, 1227 (1923).
10. BASS, L. W. *Compt. rend. soc. biol.*, **93**, 570 (1925).
11. STEIN, G., and WEISS, J. *Nature*, **162**, 814 (1948); *J. Chem. Soc.*, 3256 (1949).
12. DALE, W. N., and DAVIS, J. V. *Nature*, **163**, 64, (1949); DALE, W. N., DAVIS, J. V., and GILBERT, C. W. *Biochem. J.*, **45**, 93 (1949).
13. SCHOLES, G., and WEISS, J. *Nature*, **167**, 693 (1951).
14. CAVALLINI, D., FRONTALI, N., and TOSCHI, G. *Ibid.*, **163**, 568 (1949).
15. FRIEDEMANN, T. E., and HAUGEN, G. E. *J. Biol. Chem.*, **147**, 415 (1947).
16. PARNAS, J. K. *Biochem. Z.*, **274**, 158 (1934).

The Adsorption of Manganese by Algal Polysaccharides[1]

Arthur L. Pollard and Peter Byrd Smith

Departments of Chemical Engineering and *Bacteriology, University of Tennessee, Knoxville*

During the course of an investigation into the source of manganese deposits on the walls of a hydraulic tunnel, it was found that the initial layer consisted of partly decomposed mucilaginous polysaccharides. This was determined by installing microscope slides in a by-pass cell and analyzing the deposit by means of the Dreywood anthrone reagent (*1, 2*). After the slides had been exposed for 2 months manganese was readily detected by hydrolyzing the deposit with sulfuric acid and determination by the periodate method (*3*). Small quantities of manganese were detected in the water by the same method.

It was assumed that the polysaccharides came from decomposing algae, since fragments of algae and diatoms were found on the slides along with masses of gelatinous material showing vestiges of structure. This led to a search for an alga in a form suitable to quantitative investigation of the manganese-adsorbing mechanism. Thanks to A. J. Sharp, of the University of Tennessee Botany Department, a source of *Nostoc commune* was found which served the purpose admirably. This organism grows in colonies on bare

[1] This investigation was supported, in part, through a grant from the Aluminum Company of America.