- Jeener, R. & Rosseels, J. (1953). *Biochim. biophys. Acta*, **11**, 438.
- Kidder, G. W. & Dewey, V. C. (1949). *J. biol. Chem.* **179**, 181.
- Kidder, G. W., Dewey, V. C., Parks, R. E. & Woodside, G. L. (1949). *Science*, **109**, 511.
- Kidder, G. W., Dewey, V. C., Parks, R. E. & Woodside, G. L. (1951). *Cancer Res.* **11**, 204.
- Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
- Lasnitzki, I., Matthews, R. E. F. & Smith, J. D. (1954). *Nature, Lond.*, **173**, 346.
- Law, L. W. (1950). *Cancer Res.* **10**, 186.
- Markham, R., Matthews, R. E. F. & Smith, J. D. (1954). *Nature, Lond.*, **173**, 537.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Markham, R. & Smith, J. D. (1952a). *Biochem. J.* **52**, 552.
- Markham, R. & Smith, J. D. (1952b). *Biochem. J.* **52**, 558.
- Markham, R. & Smith, J. D. (1952c). *Biochem. J.* **52**, 565.
- Marshak, A. & Vogel, H. J. (1950). *Fed. Proc.* **9**, 85.
- Matthews, R. E. F. (1953). *J. gen. Microbiol.* **8**, 277.
- Matthews, R. E. F. (1954). *J. gen. Microbiol.* **10**, 521.
- Matthews, R. E. F. (1955). *Virology*, **1**, 165.
- Matthews, R. E. F. (1956). *Biochim. biophys. Acta*, **19**, 559.
- Matthews, R. E. F. & Smith, J. D. (1954). *Biochem. J.* **57**, vi.
- Matthews, R. E. F. & Smith, J. D. (1956). *Nature, Lond.*, **177**, 271.
- Powell, J. F. & Hunter, J. R. (1956). *Biochem. J.* **62**, 381.
- Roblin, R. O., Lampen, J. O., English, J. P., Cole, Q. P. & Vaughan, J. R. (1945). *J. Amer. chem. Soc.* **67**, 290.
- Sevag, M. G., Lackman, D. B. & Smolens, J. (1938). *J. biol. Chem.* **124**, 425.
- Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E. & Eakin, R. E. (1947). *J. Amer. chem. Soc.* **69**, 725.
- Smith, J. D. & Wyatt, G. R. (1951). *Biochem. J.* **49**, 144.
- Sugiura, K., Hitchings, G. H., Cavalieri, L. F. & Stock, C. C. (1950). *Cancer Res.* **10**, 178.
- Witkin, E. M. (1951). *Cold. Spr. Harb. Symp. quant. Biol.* **16**, 357.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
- Youngner, J. S., Ward, E. N. & Salk, J. E. (1950). *Proc. Soc. exp. Biol., N. Y.*, **75**, 157.
- Zamenhof, S. & Griboff, G. (1954). *Nature, Lond.*, **174**, 305.
- Zamenhof, S., Reiner, B., Giovanni, R. de & Rich, K. (1956). *J. biol. Chem.* **219**, 165.

Adenine Succinic Acid and Adenylsuccinic Acid from Mammalian Liver: Isolation and Identification

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Hurlbert, Schmitz, Brumm & Potter (1954) have described the chromatography on Dowex columns of purine and pyrimidine derivatives occurring in acid extracts of rat liver. The resolving power of the systems used was considerable, and the separation of a large number of substances was demonstrated. Many of these were characterized, such as mono-, di- and tri-phosphorylated derivatives of guanosine, cytidine and uridine, which appear to be analogous to those of adenosine. Other compounds, including a group of substances containing uridine diphosphate and carbohydrate, were identified tentatively. However, there were a number of compounds which were not investigated beyond determination of the spectrum and phosphate and ribose content, since in most cases they were present in amounts too small for further analysis.

Purine and pyrimidine derivatives have, in recent years, been shown to be constituents of several highly active metabolic intermediates. Examples are: guanosine diphosphate mannose (Cabib & Leloir, 1954); uridine diphosphate glucose (Caputto, Leloir, Cardini & Paladini, 1950), and related compounds; cytidine diphosphate choline

(Kennedy & Weiss, 1955) and cytidine diphosphate ribitol (Baddiley, Buchanan, Carss & Mathias, 1956); *S*-adenosylmethionine (Cantoni, 1953) and adenosine 3'-phosphate 5'-phosphosulphate (Robbins & Lipmann, 1956). Many more compounds of this type probably occur. The chromatographic procedure of Hurlbert *et al.* (1954) is well adapted for the isolation of such substances, and it is not unlikely that some of the compounds detected but not identified by them are purine and pyrimidine derivatives of the type discussed. Thus there would appear to be a need for much further work on the identification of these substances, which occur in traces only.

The isolation and characterization of two ultra-violet-light-absorbing substances occurring in small amounts in acid extracts of mouse and rabbit liver is reported in this paper. They are adenine succinic acid [6-(1:2-dicarboxyethylamino)purine] and adenylsuccinic acid [6-(1:2-dicarboxyethylamino-9-ribo-5'-phosphate)purine]. These two substances are related in the same way as adenine and 5'-adenylic acid (AMP-5'). No nucleoside corresponding to these compounds was detected. Adenine succinic acid has

not previously been reported as occurring in biological material; adeny succinic acid was found by Carter & Cohen (1955) to be formed from AMP-5' and fumaric acid in yeast autolysates, and was shown by Lieberman (1956*a*) to be an intermediate in the transformation of 5'-inosinic acid (IMP-5') to AMP-5' in extracts of *Escherichia coli*.

A preliminary report of this work has appeared (Joklik, 1956).

METHODS

Chromatography

Preparation of material for chromatography. Rabbits were killed by injecting 3 ml. of saturated MgSO_4 into the ear vein. The livers were then removed as rapidly as possible and each was placed into 150 ml. of ice-cold HClO_4 (0.6 M) in a Waring Blendor. After blending for 30 sec., the homogenates were stored at 4° until the required number of livers had been removed. The homogenates were then centrifuged, the precipitate was extracted with 2 vol. of 0.2 M- HClO_4 and the extracts were combined, neutralized to pH 6–7 with KOH and stored in the cold. The precipitated KClO_4 was filtered off after 20 hr.

Mice were killed by cervical dislocation, and the livers removed and dropped into liquid air. When the required number of livers had been collected the liquid air was removed and the livers were homogenized in HClO_4 as described above.

Since the two compounds investigated occur only in very small amounts, it was necessary to work with large batches of liver; usually ten rabbit livers or 300 mouse livers were worked up during one preparation. This resulted in about 1.5 l. of extract. In order to remove the still considerable amount of KClO_4 , which would reduce the capacity of the ion-exchanger used for the subsequent chromatography, the extract was concentrated ten times *in vacuo* and then thoroughly chilled to precipitate the perchlorate as completely as possible.

Several experiments were carried out with HClO_4 extracts which had been hydrolysed with N- HClO_4 for 30 min. at 100°. It was essential to carry out the hydrolysis before concentration of the extract, as acid treatment of the concentrated extract led to extensive darkening, possibly owing to interaction of carbohydrate and amino acids (humin formation). When such material was applied to columns, high blanks were experienced and coloured material was constantly eluted from the columns.

Chromatography on Dowex columns. The resin used was Dowex-1 (X-8) (chloride), 200–400 mesh (The Dow Chemical Co., Midland, Michigan). This was converted into the formate form; gradient elution was used. The arrangement of apparatus, as well as the techniques of using the columns and regenerating the resin, has been described in detail by Hurlbert *et al.* (1954).

Three chromatographic systems were used. The first two were the formic acid and the ammonium formate systems of Hurlbert *et al.* (1954); the third was a combination of an 'alkaline ammonium formate system' and the formic acid system, and was used for the chromatography of acid-hydrolysed liver extracts. In this system the starting eluent was 0.1 M-ammonium formate brought to pH 10 with ammonia; the reservoir contained initially 0.1 M-ammonium

formate brought to pH 4.0 with formic acid. After ten bed volumes of eluent had passed through the column this was changed to N-formic acid, and after a further five bed volumes to 4 N-formic acid. The size of the fractions collected (5–50 ml.) was varied from column to column and depended on the resolution aimed at. The eluate was stored in the cold until analysed.

Rechromatography. Fractions eluted by means of one chromatographic system were often rechromatographed by another system. As a rule the first system was the formic acid system. In this case the eluent was removed by means of freeze-drying. After chromatography by means of the ammonium formate system, either the eluent was removed by freeze-drying and sublimation of the ammonium formate *in vacuo* at 50°, or the substances to be investigated were adsorbed on Norit, after which they were eluted with ethanol–water–ammonium hydroxide (5:4:1).

Paper chromatography. All paper chromatography was by upward displacement. The solvent systems used were: (i) phenol–water (Consden, Gordon & Martin, 1944); (ii) pyridine–acetic acid–water (Decker & Riffart, 1950). Ultraviolet-light-absorbing compounds were made visible by means of a Mineralight lamp (Ultra-Violet Products Inc., South Pasadena, California) with maximum emission at 250 m μ , and amino acids by means of dipping into ninhydrin in isopropanol, followed by heating.

Paper electrophoresis. Paper electrophoresis was carried out in shallow Perspex trays, CCl_4 being used to prevent evaporation of buffer. The potential gradient used was about 8 v/cm. The pH of the buffer was varied as required according to the separation desired. Between pH 2.5 and 6 citrate buffer was used; at more acid pH and in the alkaline region glycine buffers were employed. The concentration was usually 0.1 M, though equally good results were obtained when it was increased; this was sometimes necessary when samples containing considerable amounts of salts were treated. Unwashed strips, 1 cm. wide, of Whatman no. 3 MM paper were used. Regions where compounds to be investigated had been localized were cut out and eluted with 0.1 N-HCl.

Analytical procedures

Measurement of ultraviolet-light absorption. A model DU Beckman spectrophotometer was used. Readings were always made at pH 1–2, at 260 and 275 m μ ; the ratio E_{275}/E_{260} was extensively used in the interpretation of chromatograms.

Throughout this work quantities of ultraviolet-light-absorbing materials are expressed as DU (density units); 1 DU represents that amount of material which when dissolved in 1 ml. gives an optical density of 1.000 in the Beckman spectrophotometer when the light path is 1 cm. Spectrophotometric data for naturally occurring purines and pyrimidines, and their nucleosides and nucleotides, were taken from Chargaff & Davidson (1955).

Phosphate. Phosphate was determined by the method of Fiske & Subbarow (1925), with 1-amino-2-naphthol-4-sulphonic acid as the reducing agent, and the final volume 2.0 ml. The colour was measured at 660 m μ . Acid-labile phosphate was that hydrolysed with N-acid at 100° in 10 min. Total phosphate was estimated after digestion with HClO_4 at 150–170°. The samples were then neutralized with NaOH, after which the procedure described above was followed.

Ribose. The modification by Hurlbert *et al.* (1954) of the Mejsbaum procedure was used.

Hunter's reaction for thymine. The reaction was carried out as described by Hunter (1936) except that the volumes were decreased by 40% throughout.

Test for diazotizable amines. This test was carried out by a modification of the method of Ravel, Eakin & Shive (1948). To the sample (10–100 μ g. of amine) were added 1 ml. of $N-HCl$, 1 ml. of ethanol and 0.5 ml. of 0.1% $NaNO_2$. After 10 min. 0.5 ml. of 0.5% ammonium sulphamate was added; after 3 min. this was followed by 0.5 ml. of 0.1% *N*-ethylnaphthylamine. The colour developed was read at 520 $m\mu$ after 20 min.

Preparative

Formic acid hydrolyses. Hydrolyses with 90% formic acid were carried out (i) at 170° for 30 min. in order to hydrolyse the base-ribose bond of nucleotides, and (ii) at 180° for 1 hr. in order to decompose adenine succinic acid. Since hydrolysis in sealed glass tubes is rather troublesome, this

was carried out in open tubes inserted into a small bomb (Fig. 1) which was placed in a paraffin bath. The bomb was constructed of stainless steel; the sealing surfaces were lightly coated with silicone grease. The bomb proved to be gas-tight at the considerable pressures built up at 180°; the gases escaped quietly on opening, without loss of formic acid from inside. After hydrolysis the samples were prepared for chromatography or electrophoresis as described by Wyatt & Cohen (1953).

Decomposition of adenine succinic acid by hydrolysis with barium hydroxide. Adenine succinic acid (synthetic, 40 mg.) was refluxed with 5 ml. of saturated $Ba(OH)_2$ for 20 hr. The reaction mixture was then diluted to 100 ml. and carefully neutralized with H_2SO_4 . The precipitated $BaSO_4$ was removed and the solution evaporated.

Synthesis of adenine succinic acid. Carter (1956) has briefly outlined the synthesis. The procedure employed here was as follows: 2.33 m-moles of 6-chloropurine (prepared by the method of Bendich, Russell & Fox (1954)) were dissolved in 40 ml. of water and 4 m-moles of L-aspartic acid added. The pH was adjusted to 9.5 with NaOH, and the mixture refluxed. The course of the reaction was followed by changes in E_{275}/E_{260} . The ratio started at 0.62 and had risen to 1.00 after 1 hr. and to 1.39 after 3 hr. The reaction mixture was then added to 200 ml. of ethanol, and left at -20° for 24 hr. An oil settled out; the supernatant was decanted and again diluted threefold with ethanol; after chilling, more oil separated out. The oil readily solidified on scratching. The combined deposits were taken up in water; E_{275}/E_{260} was 1.56 at pH 1. The product was then chromatographed on Dowex-1 (formate), the formic acid system being used. The bulk of the material was eluted as a single band, with E_{275}/E_{260} about 1.67. Eluent was removed by freeze-drying the yield was 1.3 m-moles, assuming a mol.wt. of 251. The material was recrystallized first from ethanol, and then from water. ϵ_{276} at pH 1 was 13.7×10^3 (Found: C, 42.8; H, 3.7; O, 25.75; N, 27.2; $C_8H_8O_4N_6$ requires C, 43.0; H, 3.6; O, 25.5; N, 27.9%).

Preparation of 3'-nucleotidase. This enzyme was prepared from 1 lb. of rye-grass seed by the method of Shuster & Kaplan (1953). Purification was carried as far as the elution from alumina C_7 and subsequent dialysis. At this stage the enzyme preparation was without activity on 2'-adenylic acid (AMP-2'), and attacked 3'-adenylic acid (AMP-3') 10 times faster than AMP-5'. The enzyme was stored at -20° ; it retained its activity for at least one year.

Preparation of 5'-nucleotidase. This enzyme was prepared from bull semen (kindly provided by Dr I. G. White, Department of Veterinary Physiology, University of Sydney) by the simplified procedure of Heppel & Hilmoe (1951). The only isomer of AMP attacked at a measurable rate was AMP-5'. Adenosine di- and triphosphate (ADP and ATP) were not attacked. The enzyme was stored at -20° and retained its activity for over two years.

Dephosphorylation of adenylysuccinic acid. Adenylysuccinic acid (135 DU) was incubated with 0.1 M-aminotrihydroxymethylmethane buffer, pH 7.0 (3.0 ml.), M-MgSO₄ (0.1 ml.), and 5'-nucleotidase (0.6 ml.) at 20° overnight. The reaction mixture was deproteinized by the addition of trichloroacetic acid to a final concentration of 5%, and adenosine succinic acid adsorbed on Norit. It was eluted as described above and subjected to paper electrophoresis to check the purity.

Fig. 1. Steel bomb for formic acid hydrolyses. The bomb consists of: a stainless-steel cylinder A into which is placed the open glass tube containing the sample to be hydrolysed; a stainless-steel cap B; and a mild-steel stirrup C by means of which B is firmly clamped to A.

RESULTS

Chromatography of perchloric acid extracts of mouse and rabbit liver

Fig. 2 shows the chromatogram obtained when the perchloric acid extract of ten rabbit livers was subjected to chromatography on a Dowex-1 (formate) column. Compounds were identified by reference to

Fig. 2. Chromatography of an HClO_4 extract of rabbit liver by means of the formic acid system. Total number of density units (DU) applied to the column, 55000. The mixing flask contained 400 ml. of water, the reservoir 4N-formic acid. The number of DU in the fractions which were too concentrated to be graphed completely were 60, 213, 352 and 68. The substances marked on the graph were identified in the course of this work; the ones in parentheses were reported by Hurlbert *et al.* (1954) to occur in the places indicated. The position of the substances within a peak is indicated approximately on the graph, e.g. the position of CDP indicates that this compound was found in the ascending portion of the third major peak. DPN, TPN and GMP refer to di- and triphosphopyridine nucleotide and guanylic acid respectively; other abbreviations are explained in the text.

Fig. 3. Rechromatography of the substances in the UMP region of Fig. 2 by means of the ammonium formate system. Total number of DU applied to the column, 2700. The mixing flask contained 500 ml. of water, and the reservoir M-ammonium formate. The latter was changed to 2M-ammonium formate after 500 ml. of eluate had been collected. The number of DU in the fractions which were too concentrated to be graphed completely were: (UMP) 7.8, 11.0, 18.0, 17.5, 5.5; (IMP) 12.5, 12.8, 7.6, 6.3. The material with E_{275}/E_{260} of between 1.0 and 1.2 occurs in the last two peaks.

columns on which mixtures of known substances had been chromatographed and identified in the eluate fractions by their characteristic spectra. The order of elution found in this work agreed with that reported by Hurlbert *et al.* (1954). Chromatography may be continued beyond the point shown in Fig. 2, but as the substances studied had eluted by the time ADP appeared, this was taken as a convenient, easily recognizable end point. The pattern of elution was highly reproducible, as were the relative proportions of the major constituents. Chromatograms from mouse livers were similar except for a decreased cytidine diphosphate (CDP)–inosinic acid (IMP)–uridylic acid (UMP) band.

It was noted that in the descending region of this band the ratio E_{275}/E_{260} rose to about 0.8. This occurred when CDP had already been eluted off the column, and no reference compounds that were used gave rise to this phenomenon in this region. On rechromatography of the whole band by means of the ammonium formate system, the chromatogram shown in Fig. 3 was obtained. The first band was identified as UMP, the second as IMP, on the descending shoulder of which the ratio E_{275}/E_{260} changed to over 1, indicating a cytosine compound, most probably, from its position on the chromatogram, CDP; then came a small amount of ADP, and then a double band with a ratio E_{275}/E_{260} of 0.9–1.2. This was the last band eluted.

Preliminary tests showed that the substances eluted in these last two bands did not comprise any of the known purine or pyrimidine derivatives. Further work was directed at their isolation and identification.

The total amount of material giving this high ratio E_{275}/E_{260} was very small; with the ten rabbit

Fig. 4. Chromatography of an acid-hydrolysed HClO_4 extract of rabbit liver by means of the alkaline ammonium formate–formic acid system. Total number of DU applied to the column, 50 000. The mixing flask contained 800 ml. of 0.1M-ammonium formate (pH 10), and the reservoir 0.1M-ammonium formate (pH 4), which was changed to M-formic acid after 1200 ml. of eluate had been collected, and again to 4M-formic acid after 1800 ml. The number of DU in the fractions which were too concentrated to be graphed completely were: 70, 84, 80 and 53.

livers, the total amount of material in the perchloric acid extract absorbing at 260 $m\mu$ was 55 000 DU, of which 150–200 proved to be the new material. It was found that in order to isolate enough material for recrystallization and elementary analysis it was more practicable to run a series of two-column systems as has just been described rather than to perform one big separation.

Several experiments were done using acid-hydrolysed perchloric acid extract (see Methods). The aim was to convert all purine-containing nucleotides, which make up the bulk of the acid-soluble, ultraviolet-light-absorbing substances, into the free bases. As a result, the chromatograms were greatly simplified (Fig. 4). Among the bands identified were adenine (A), guanine (G) and cytidylic acid (CMP); then came a complex band containing the material with E_{275}/E_{260} of about 1.0, which was further resolved by means of the ammonium formate system as above.

Identification of adenine succinic acid

Material with E_{275}/E_{260} of 0.8 and above was freed from eluent either by sublimation of ammonium formate or by adsorption on to Norit followed by elution with ethanol–water–ammonia. On electrophoresis in 0.1M-citrate buffer, pH 3.0, two components were recognized, the relative amounts of which were about 1.5:1. This ratio was the same for mouse and rabbit liver. The first major component carried a negative charge and had the following spectrum: maximum (pH 1) at 267 $m\mu$; E_{275}/E_{260} , 0.95. The other, which carried a small positive charge, had a maximum (pH 1) at 276 $m\mu$ and an E_{275}/E_{260} ratio of 1.65. The first component was transformed into the second by hydrolysis with N-HCl at 100° for 30 min. Furthermore, whereas the first contained ribose and phosphate, the second contained neither. It thus appeared likely that on hydrolysis the nucleotide-like first component was hydrolysed to the second, which was the simpler of the two. Evidence that this second compound is adenine succinic acid is presented in the following sections.

Chemical evidence. (a) On hydrolysis with 90 % formic acid at 180° for 1 hr. the spectrum changed. When the hydrolysis mixture was subjected to electrophoresis, adenine and hypoxanthine were identified by comparison with authentic samples. Table 1 shows that, under the conditions of hydrolysis used, adenine itself decomposed to hypoxanthine to a small extent, but not enough to account for all the hypoxanthine formed from the unknown substance. It was clear that the major pathway of breakdown led to hypoxanthine, but that some adenine was also formed. (b) On hydrolysis with saturated baryta at 100° for 20 hr., a ninhydrin-positive substance was liberated. This substance was

Table 1. *Formic acid hydrolysis of adenine and adenine succinic acid*

The conditions of hydrolysis are described under Methods. Electrophoresis of the hydrolysate was first carried out at pH 2.7 for 4 hr.; the composite adenine succinic acid–hypoxanthine spot was then again subjected to electrophoresis, at pH 6. Substances were estimated spectrophotometrically.

Compound hydrolysed	Substances identified in hydrolysate
Adenine (0.52 μ mole)	Adenine (0.45 μ mole) Hypoxanthine (0.05 μ mole)
Adenine succinic acid (0.65 μ mole)	Adenine succinic acid (0.06 μ mole) Hypoxanthine (0.42 μ mole) Adenine (0.11 μ mole)

identified as aspartic acid on the basis of its chromatographic behaviour in phenol–water and pyridine–acetic acid–water. (c) The test of Koritz & Skoog (1952) for purines was positive. (d) On boiling with 6N-H₂SO₄ for 30 min., the compound gave a positive test for diazotizable amine. Neither adenine nor guanine gave this test after similar treatment.

The results of these tests are consistent with the hypothesis that the compound is adenine succinic acid. On hydrolysis with formic acid the compound appears to be split in two different places, so that adenine and hypoxanthine are yielded simultaneously; on hydrolysis with baryta a similar reaction occurs and the other split product of the molecule was identified as aspartic acid. This substance could not originate from the purine skeleton. Finally, Carter (1956) has reported that adenine succinic acid is transformed by treatment with boiling acid into a substance giving a diazotizable amine test.

In further experiments the behaviour of material isolated from liver was compared with that of synthetic adenine succinic acid (see Methods).

Analysis of the spectrum. The spectrum of the substance was compared with that of synthetic adenine succinic acid at pH values 2.1, 2.9, 3.6, 4.1, 4.6, 5.2, 5.9, 6.9, 8.3, 9.6, 10.2, 10.7, 11.1 and 11.9. The curves coincided exactly in all cases. Fig. 5 shows the spectra at pH 2.1, 6.9 and 11.9. The maximum shifts from 276 $m\mu$ at pH 2.1 to 269 $m\mu$ at pH 6.9 and back to 274 $m\mu$ at pH 11.9. Table 2 gives the values of the ratio E_{275}/E_{260} at all pH values investigated. They vary from 1.62 at pH 2.1 to 1.16 at pH 5.9 and back to 1.68 at pH 11.9. These changes in the spectrum indicate the occurrence of two dissociations. The pK values of the two dissociations were calculated from the extinction values at 260 and 275 $m\mu$ by means of the formula E_{275}/E_{260} at half dissociation = $\frac{1}{2}(a+x)/\frac{1}{2}(b+y)$, where a and x are the extinction values of the two ionic

species at 275 m μ and b and y the corresponding values at 260 m μ . In passing from acid pH to neutral pH, the pK occurs where E_{275}/E_{260} is $\frac{1}{2}(13.7 + 12.2)/\frac{1}{2}(8.45 + 10.85) = 1.365$, i.e. at pH 3.75; and in passing from neutral pH to alkaline pH, where the value of E_{275}/E_{260} is

$$\frac{1}{2}(12.6 + 12.2)/\frac{1}{2}(10.85 + 7.25) = 1.37,$$

i.e. at pH 10.5. Thus the two pK values for which there is optical evidence are those at pH 3.75 and 10.5.

Electrophoretic behaviour. The electrophoretic behaviour of adenine succinic acid at pH 3.3 and

Fig. 5. Spectrum of adenine succinic acid. Continuous curve, pH 2.1; broken curve, pH 6.9; dotted curve, pH 11.9. pH 2.1: $\lambda_{\max}$ 276 m μ , $\epsilon_{\max}$ 13.7×10^3 ; $\lambda_{\min}$ 234 m μ , $\epsilon_{\min}$ 2.09×10^3 . pH 6.9: $\lambda_{\max}$ 268 m μ , $\epsilon_{\max}$ 14.0×10^3 ; $\lambda_{\min}$ 231 m μ , $\epsilon_{\min}$ 1.74×10^3 . pH 11.9: $\lambda_{\max}$ 274 m μ , $\epsilon_{\max}$ 12.2×10^3 ; $\lambda_{\min}$ 240 m μ , $\epsilon_{\min}$ 2.50×10^3 .

Table 2. Adenine succinic acid: E_{275}/E_{260} at different pH values

pH	2.1	2.9	3.6	4.2	4.6	5.2	5.9	6.9
E_{275}/E_{260}	1.62	1.55	1.39	1.30	1.27	1.23	1.16	1.16
pH	8.3	8.8	9.6	10.2	10.7	11.0	11.9	
E_{275}/E_{260}	1.17	1.19	1.20	1.30	1.43	1.55	1.68	

Table 3. Electrophoretic behaviour of adenine succinic acid

pH	Buffer	Distance travelled/hr. (mm.)	Charge carried
2.0	Glycine	26	+
2.8	Citrate	6	+
3.0	Citrate	3	+
3.3	Citrate	0	0
3.6	Citrate	11	-
6.6	Citrate	65	-

6.0 has been compared with that of adenine, AMP-5' and adenylysuccinic acid (Joklik, 1956). Table 3 lists in more detail the distances travelled at pH values around 3.3, where adenine succinic acid reverses its charge. Once again the substance isolated from liver showed behaviour at all pH values identical with that of the synthetic substance. It was found that at pH 2 the substance carried a positive charge; that at pH 3.3 it was uncharged; and that at pH 3.6 it had a slight negative charge. Above pH 4.5 the substance carried at least one and possibly more than one negative charge. These findings and their correlation with the pK values found by the spectrophotometric technique are discussed below (see Discussion).

Preparation of liver adenine succinic acid for elementary analysis. Several batches of liver extract were chromatographed on Dowex-1 (formate) columns with both the formic acid and the ammonium formate system, as described above, until about 1000 DU had been accumulated. After the removal of ammonium formate by sublimation, the material was hydrolysed for 30 min. in $N-H_2SO_4$ at 100° so as to break down adenylysuccinic to adenine succinic acid. While the mixture was still warm, silver nitrate was added. The precipitate was collected, washed with ice-cold H_2SO_4 , and extracted with hot 0.1N-HCl. The precipitation and extraction were repeated twice more, and the material was subjected to chromatography on a Dowex-1 (formate) column with the formic acid system. The material eluted in a very sharp band, the ratio E_{275}/E_{260} of which was 1.65 at pH 1. On lyophilization a white powder was obtained, yield 8.3 mg. This substance, however, appeared to be a salt of adenine succinic acid, as it dissolved readily in water. Similar behaviour was shown by synthetic adenine succinic acid as obtained by column chromatography. When recrystallized from ethanol and then from water a material was obtained which was very sparingly soluble in cold water. The material derived from liver was thus also recrystallized from water and yielded 6.1 mg. of white crystals. The value of ϵ_{276} at pH 1 was 13.7×10^3 , the same value as found for the synthetic material (Found: C, 42.5; H, 3.7; O, 26.0; N, 27.1; $C_9H_9O_4N_5$ requires C, 43.0; H, 3.6; O, 25.5; N, 27.9%).

It was concluded on the evidence presented that the substance is adenine succinic acid.

Identification of adenylysuccinic acid

As mentioned above, a substance containing adenine succinic acid, ribose and phosphate was associated with adenine succinic acid in the eluate from Dowex columns. Table 4 shows that there were present in the molecule 1 mol. of phosphate, 1 mol. of ribose, and 1 mol. of adenine succinic acid. The ribose-adenine succinic acid bond was hydrolysed

by heating at 100° in *N*-HCl for 30 min. The position of the phosphate group was determined by enzymic analysis: no phosphate was split off by 3'-nucleotidase, whereas approximately 1 mol. of phosphate was split off by 5'-nucleotidase. In all these reactions the compound reacted in exactly the same manner as a sample of adenylysuccinic acid kindly furnished by Dr Carter. Furthermore, spectra of the two compounds were identical (Fig. 6). Electrophoretic movement at three pH values was identical: at pH 2.8, 3.1 and 6.0 the substances moved 20, 20 and 58 mm./hr. respectively. It was concluded on this evidence that the compound is adenylysuccinic acid.

Table 4. *Composition of adenylysuccinic acid*

Group analysed	Amount present (μ moles/16.9 DU*)
Ribose	0.96
Acid-labile phosphate	0
Total phosphate	0.93
3'-Phosphate	0
5'-Phosphate	0.91
Adenine succinic acid†	0.98

* The value reported by Carter & Cohen (1955), i.e. $\epsilon = 16.9 \times 10^3$ at pH 1, was used.

† Analysis by spectrum after hydrolysis with *N*-HCl at 100° for 30 min.

Fig. 6. Spectrum of adenylysuccinic acid. Continuous curve, pH 1; broken curve, pH 6.75; dotted curve, pH 13. The values for ϵ were determined by reference to adenine succinic acid. They are 2–3% lower than those of Carter & Cohen (1955). pH 1: $\lambda_{\max}$. 267 $m\mu$, $\epsilon_{\max}$. 16.6×10^3 ; $\lambda_{\min}$. 234 $m\mu$, $\epsilon_{\min}$. 3.28×10^3 . pH 6.75: $\lambda_{\max}$. 269 $m\mu$, $\epsilon_{\max}$. 17.6×10^3 ; $\lambda_{\min}$. 232 $m\mu$, $\epsilon_{\min}$. 2.14×10^3 . pH 13: $\lambda_{\max}$. 269 $m\mu$, $\epsilon_{\max}$. 18.5×10^3 ; $\lambda_{\min}$. 233 $m\mu$, $\epsilon_{\min}$. 2.6×10^3 .

Stability of adenylysuccinic acid

There was the possibility that adenylysuccinic acid was unstable, and that adenine succinic acid was formed during the isolation procedure. The stability of adenylysuccinic acid during the various steps of the isolation was therefore investigated, among them the acid extraction step, chromatography on Dowex columns and the sublimation of ammonium formate. Adenylysuccinic acid was stable under all conditions employed, so that it was concluded that both the free purine and the nucleotide occur in liver.

Probable absence of adenosine succinic acid

No trace of the nucleoside corresponding to adenylysuccinic acid was detected in the biological material. It was therefore determined whether this material, if present in the ammonium formate eluate, would be detected subsequently in the electrophoretic separation. Adenylysuccinic acid was dephosphorylated by means of 5'-nucleotidase, the adenosine succinic acid adsorbed on Norit and eluted as described above, and then subjected to electrophoresis at pH 2.8. It was found that at this pH the nucleoside carried a small negative charge. It was well separated from both the free purine and the nucleotide, and the separation of all three compounds could be accomplished on one strip. Since no spot was observed in the position occupied by the nucleoside, it was concluded that this compound either does not occur in liver in detectable amounts or is eluted in a different position. Since the free purine and the nucleotide were eluted by both the formic acid and the ammonium formate systems in closely adjacent positions, the second possibility seemed unlikely.

Metabolism of adenine succinic acid and adenylysuccinic acid in liver homogenates

It was of interest to determine whether either of these compounds is metabolized by liver homogenates if added in substrate concentrations. Homogenates (15%) of mouse liver in a medium containing sodium chloride (0.1M), phosphate (0.02M, pH 7.6) and magnesium chloride (0.003M) were used. Adenine succinic acid was not metabolized over a period of 5 hr. at 35°, but within this period adenylysuccinic acid was completely converted into the nucleoside. The nucleoside was identified by its electrophoretic mobility, spectrum (which was found to be the same as that of the nucleotide at pH 1), absence of phosphate and presence of ribose. It appears, however, since no nucleoside was detected in acid extracts of liver, that this dephosphorylation occurs extremely slowly if at all in the intact liver, where the concentration of nucleotide is of the order of one-thousandth of that used here.

Reaction with diazotized sulphanilic acid

Hunter (1936) has described a reaction of thymine with diazotized sulphanilic acid. This reaction proceeds in alkaline medium, and in the presence of hydroxylamine yields a highly coloured complex. Adenine, hypoxanthine, uracil and cytosine do not react, and guanine yields a coloured complex in the absence of hydroxylamine (W. K. Joklik, unpublished results). It was found that adenine succinic acid also forms a coloured complex, under the same conditions as thymine, i.e. only in the presence of hydroxylamine. The spectrum of this complex is identical with that produced by thymine, and different from that produced by guanine. Adenylsuccinic acid and adenosine succinic acid do not produce this complex; on acid hydrolysis, the liberated adenine succinic acid reacts. It was noted that if adenine succinic acid is pretreated with acid (N-acid at 100° for 30 min.) there is a 100 % increase in the colour yield; the reason for this is obscure. With thymine, pretreatment with acid led to a much smaller (about 20 %) increase in the intensity of the colour produced.

DISCUSSION

The isolation of adenine succinic and adenylsuccinic acid from liver raises the question of the function of these two compounds in cellular metabolism. With adenylsuccinic acid the position is clearer than with the free purine. Lieberman (1956*a*) has shown that an enzyme system in *E. coli* carries out the following reaction: IMP-5' + aspartic acid + guanosine triphosphate → adenylsuccinic acid + guanosine diphosphate. According to Lieberman (1956*b*) a derivative of IMP-5' phosphorylated at C6 may be an intermediate. Furthermore, another enzyme splits adenylsuccinic acid:

The reversible cleavage of adenylsuccinic acid to AMP-5' and fumaric acid was also demonstrated in yeast by Carter & Cohen (1955).

It is thus possible that adenylsuccinic acid represents an intermediate in the transformation of IMP-5' to AMP-5'. The above reactions were demonstrated in extracts from micro-organisms; the finding of adenylsuccinic acid in liver suggests that a similar pathway exists in mammalian tissue. It is noteworthy that only the 5'-nucleotides were found to participate in the above reactions, and that the compound isolated from liver is likewise the 5'-isomer.

The occurrence of adenine succinic acid is more difficult to explain. One possibility is that it is merely a breakdown product of adenylsuccinic acid. When adenylsuccinic acid was incubated with a liver homogenate, it was dephosphorylated to the

nucleoside, but in view of the absence of the nucleoside in acid-soluble fraction of liver it appears that this reaction proceeds slowly within the intact cell, if at all. Furthermore, the nucleoside accumulated, so that the conversion of nucleoside into free purine proceeded more slowly than the dephosphorylation. However, it is conceivable that there exists a transglycosidation mechanism, so that in the presence of a suitable ribose acceptor the transformation of nucleoside into free purine may be more rapid and of some importance.

A second possibility is that adenine succinic acid represents an intermediate between hypoxanthine and adenine, like adenylsuccinic acid in the case of the nucleotides. Although there is evidence that the free purines are not precursors of nucleotides (Greenberg, 1951), there is present within the cell a mechanism for the conversion of both adenine (Safran & Scarano, 1953) and hypoxanthine (Williams & Buchanan, 1953) into their nucleotides. The existence of this purine-activating mechanism indicates that there may be a second pathway to AMP-5' in addition to that through IMP-5' in which hypoxanthine may or may not be a precursor; and this is the path from hypoxanthine to AMP-5' via adenine, in which adenine succinic acid may well be an intermediate.

An interesting point is the relative amount of adenine succinic and adenylsuccinic acids found in liver. Whereas these two compounds occur in nearly equal concentration, adenine, guanine, cytosine and uracil occur in amounts very much less than their nucleotide forms.

Spectrophotometric measurements at different pH values showed that adenine succinic acid has at least two dissociations. That found in the alkaline region, at pH 10.5, is almost certainly due to the same ionization as in adenine, namely the removal of a proton from the imidazole ring of the neutral molecule (Bendich, 1955). In the acidic region the following ionizations are possible: the dissociations of the two acidic groups of succinic acid, of the substituted amino group and of a ring nitrogen. The last dissociates only at very low pH values [cf. adenine (Johnson, 1955)]. The dissociations of the carboxyl groups of succinic acid yield ionic species which have the same spectrum as undissociated adenine succinic acid, so that the dissociation detected by spectrophotometric measurements is that of the substituted amino group. The value found, *pK* 3.75, is slightly lower than that of adenine (4.1, Cohn, 1955), which would be expected on theoretical grounds. As for the evidence from electrophoretic studies, it appears that at pH 3.3 the dissociations of the first carboxyl group of the succinic acid moiety and of the substituted amino group are equal, so that the molecule as a whole is uncharged and therefore does not migrate.

The chromatographic behaviour of the two substances is of interest. Adenylsuccinic acid behaves as expected: in the formic acid system it is eluted just ahead of ADP, for in formic acid the phosphate group and rather less than one of the two succinic carboxyl groups are dissociated. Similarly, in the ammonium formate system it is eluted just behind ADP, for now the phosphate group and rather more than one of the two succinic carboxyl groups are dissociated. Adenine succinic acid, however, behaves in an anomalous manner: it closely accompanies adenylsuccinic acid in both systems. This behaviour would probably have to be ascribed to non-polar retention.

Treatment with acids and bases at elevated temperatures affects adenine succinic acid in a number of ways. Concentrated formic acid at 180° for 1 hr. hydrolyses it in such a manner that both adenine and hypoxanthine appear among the split products. Cleavage occurs on both sides of the nitrogen atom attached to C6. The other split products, which were not identified, would be expected to be fumaric acid and aspartic acid. Treatment with baryta yielded aspartic acid and, presumably, hypoxanthine. Treatment with hot mineral acids first transforms the compound into a form which yields twice the colour with Hunter's reagent as untreated adenine succinic acid; the nature of this transformation is not known. Hunter (1936) has discussed the reaction with diazotized sulphanilic acid and hydroxylamine, but the precise nature of the coloured complex formed appears to be unknown. Further treatment with mineral acid leads to the formation of a compound giving a test for a diazotizable amine; Carter (1956) has suggested that this transformation may involve a cyclization of the succinic acid moiety of the molecule.

Note. Since this paper was accepted for publication, an investigation of adenylsuccinic acid and adenylsuccinase (the enzyme catalysing its reversible cleavage into AMP and fumaric acid) has been reported (Carter & Cohen, 1956).

SUMMARY

1. Two purine derivatives were isolated from perchloric acid extracts of mouse and rabbit livers.
2. Evidence is presented that they are adenine succinic acid and adenylsuccinic acid.
3. Certain chemical properties, the spectra and electrophoretic behaviour of the two substances were investigated.
4. The synthesis of adenine succinic acid is described.

5. The function within the cell of adenine succinic acid and adenylsuccinic acid is discussed.

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REFERENCES

- Baddiley, J., Buchanan, J. G., Carss, B. & Mathias, A. P. (1956). *Biochim. biophys. Acta*, **21**, 191.
- Bendich, A. (1955). In *The Nucleic Acids*, p. 114. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press.
- Bendich, A., Russell, P. J. & Fox, J. L. (1954). *J. Amer. chem. Soc.* **76**, 6073.
- Cabib, F. & Leloir, L. F. (1954). *J. biol. Chem.* **206**, 779.
- Cantoni, G. L. (1953). *J. biol. Chem.* **204**, 403.
- Caputto, R., Leloir, L. F., Cardini, C. E. & Paladini, A. C. (1950). *J. biol. Chem.* **184**, 333.
- Carter, C. E. (1956). *Fed. Proc.* **15**, 230.
- Carter, C. E. & Cohen, L. H. (1955). *J. Amer. chem. Soc.* **77**, 499.
- Carter, C. E. & Cohen, L. H. (1956). *J. biol. Chem.* **222**, 17.
- Chargaff, E. & Davidson, J. N. (1955). *The Nucleic Acids*. New York: Academic Press.
- Cohn, W. E. (1955). In *The Nucleic Acids*, p. 217. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press.
- Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
- Decker, P. & Riffart, W. (1950). *Chem. Ztg.* **74**, 261. [From *Chem. Abstr.* (1951). **45**, 6528.]
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Greenberg, G. R. (1951). *J. biol. Chem.* **190**, 611.
- Heppel, L. A. & Hilmo, R. J. (1951). *J. biol. Chem.* **188**, 665.
- Hunter, G. (1936). *Biochem. J.* **30**, 745.
- Hurlbert, R. B., Schmitz, H., Brumm, F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
- Johnson, E. A. (1955). Unpublished results, in *The Nucleic Acids*, p. 502. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press.
- Joklik, W. K. (1956). *Biochim. biophys. Acta*, **22**, 211.
- Kennedy, E. P. & Weiss, S. B. (1955). *J. Amer. chem. Soc.* **77**, 250.
- Koritz, H. G. & Skoog, F. (1952). *Arch. Biochem. Biophys.* **38**, 15.
- Lieberman, I. (1956a). *J. Amer. chem. Soc.* **78**, 251.
- Lieberman, I. (1956b). *Fed. Proc.* **15**, 301.
- Ravel, J. M., Eakin, R. E. & Shive, W. (1948). *J. biol. Chem.* **172**, 67.
- Robbins, P. W. & Lipmann, F. (1956). *J. Amer. chem. Soc.* **78**, 2652.
- Saffran, M. & Scarano, E. (1953). *Nature, Lond.*, **172**, 949.
- Shuster, L. & Kaplan, N. O. (1953). *J. biol. Chem.* **201**, 535.
- Williams, W. J. & Buchanan, J. M. (1953). *J. biol. Chem.* **203**, 583.
- Wyatt, G. R. & Cohen, S. S. (1953). *Biochem. J.* **53**, 774.