I. The Energy Relationships in the Ornithine-Citrulline-Arginine-Urea Cycle

II. Thermal Data for other Biochemicals

III. A Study of Sperm Agglutination

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SUMMARY

I. Methods for preparation and purification of arginine, citrulline, and ornithine were developed. The necessary entropies, heats of formation, solubilities, dissociation constants, activities, and vapor pressures were evaluated from the appropriate physical measurements of these compounds. These and other data were combined to calculate the free energy changes in each step of the ornithine-citrulline-arginine-urea cycle.

II. Methods of preparation, and purification, for other thermal data, of many biochemicals were developed. The entropies of creatine hydrate, glycyl glycine, hippuric acid, hippuryl glycine, l-proline, and taurine were evaluated from specific heat measurements. heats of combustion of fumaric and maleic acids were measured.

III. The biological properties of egg-borne marine sperm agglutinins were studied. Bio-assays were developed and active extracts were prepared. These extracts were shown to be of a protein nature. Activation and agglutination of marine sperm by various substances were studied. Correlations of the relation of molecular structure to agglutinating activity were made and discussed.

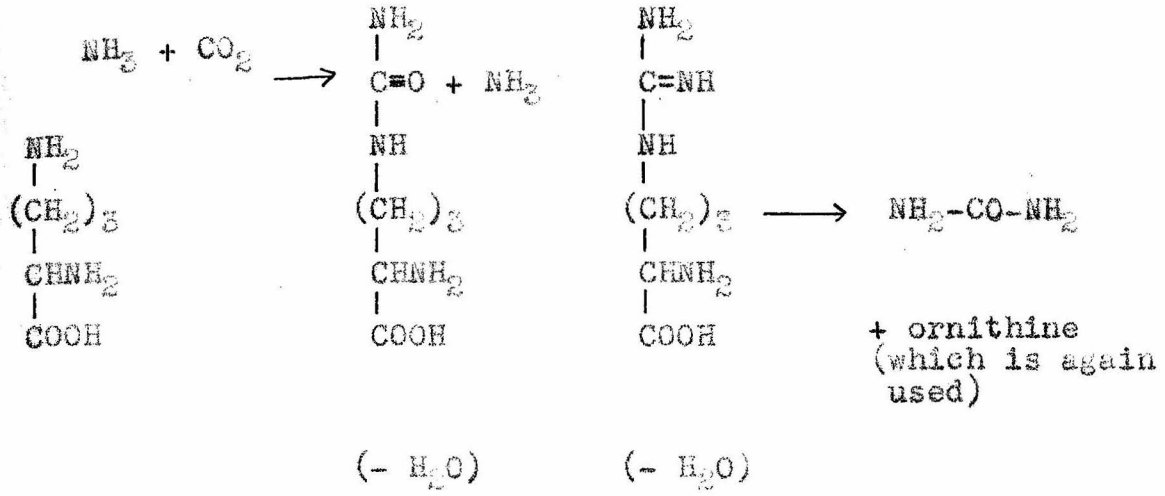
Introduction

In 1828 Wohler synthesized urea (1). This compound had been known as a constituent of mammalian urine for a considerable period before that date. It is now known that a large portion of the urea arises originally from protein and amino acids. As protein and amino acids are metabolised ammonia is first liberated from the metabolites. Since a very small concentration of ammonia in the blood is toxic to the mammal, the animal could not continue to live unless a mechanism or mechanisms for removal were operative. The evidence for the overall mechanism involving conversion of ammonia into urea in vivo was first presented by Knierim (3) and Salkowski (8) who showed that administered ammonium salts could be recovered as urea in the urine.

The steps in the biological mechanism of urea formation from ammonia then needed to be examined. Salkowski believed that the synthesis occurred in the same fashion that Wohler demonstrated for his in vitro synthesis. Drechsel (4) believed that the mechanism required ammonium carbamate as an intermediate. Neither of these methods were submitted to decisive experiments.

In 1932, Krebs and Henseleit (5) tested various compounds in an effort to find those which would facilitate urea formation in the liver slices. The compounds which were effective were arginine, citrulline, and ornithine. They

then postulated the following cycle:



Evolution of the free energy relationships of the compounds according to the procedure of Borsook and Huffman (6) provides an insight into the actual probability of occurrence of such mechanisms. The obtaining of the necessary physical data and evaluation of the derived free energy data for the above cycle was the subject of this thesis.

PREPARATION OF THE COMPOUNDS

General

Important divisions of the general problem were the preparation in large quantities (thirty - fifty grams) of pure ornithine, arginine, and citrulline. The literature included methods for the enzymatic preparation of ornithine from arginine in turn prepared by an expensive and cumbersome process;and methods for obtaining small quantities of citrulline by isolation or synthesis. Since the flavianate method (7) had been improved (8, 9) to render arginine salts readily available, it was felt that with this as a cornerstone, it would be more expeditious to develop simple, efficient methods to supplant the older impractical procedures for the preparation of the substances needed. It was further desired to avoid enzymic hydrolyses and direct isolations since these were far less likely to give the high purity materials required by precise modern calorimetry.

As an illustration of the superiority of the more strictly synthetic method, the experience with leucine might be cited. Leucine isolated from a natural source had been found to contain methionine (10), as well as isoleucine, neither impurity being removable by simple recrystallisation. This was reflected in the disagreement of heats of combustion of l-leucine samples prepared by direct isolation.

When the impurities were removed by recrystallisation of the combined formyl derivatives (11) it was possible to obtain an l-leucine, the heat of combustion of which, checked that of a resolved synthetic leucine.

d-ARGININE

Since Gulewitsch (12) and later workers (13) prepared d-arginine by an expensive, cumbersome method from the silver salt, a truly practical method for this substance was needed. One would expect that with the development of the now classical flavianate method (8) and the numerous papers describing improvements thereon that the preparation of free arginine from the flavianate or one of the salts prepared from it would have been described. It had not been; the explanation may lie in the fact that the flavianate is generally converted into hydrochloride and an unsuccessful attempt to prepare pure arginine from the hydrochloride had been recorded (14).

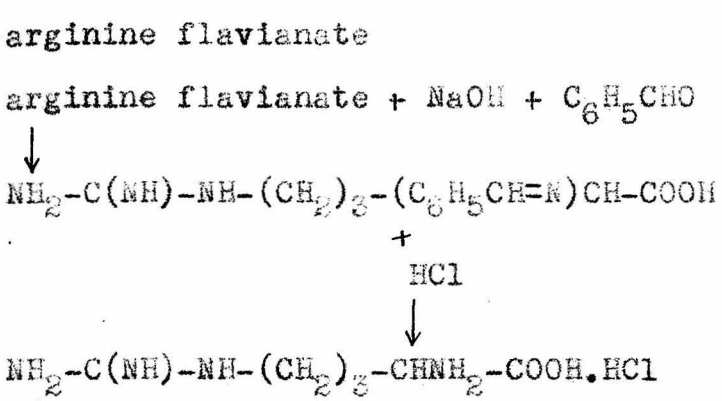
Two necessary conditions for preparation of pure crystalline arginine were found in this work. The first was that the original protein source should be one which did not offer seriously contaminating amino acids. For instance, arginine hydrochloride from casein, hog's blood, or sardine spermatic tissue could not be converted into crystalline arginine by the conventional procedure, whereas the chlorides obtained from commercial salmine or a good grade of gelatin were successfully worked up. The second requirement was the removal of excess silver from the arginine solution by hydrogen sulfide. Since an excess of silver oxide was used for removal of the chloride from the amino acidium chlorides, and siver-arginine is soluble in arginine solutions (12) this silver must be removed. By observing these precautions a local amino

acid factory was able for the first time to obtain free arginine.

The arginine was prepared and purified by the following series of reactions:

Gelatine + HCl ⟶ arginine + other amino acids

arginine flavianate



The hydrochloride was then converted to free arginine in the following way: 8.03 gm. of silver nitrate (3 percent excess over the arginine hydrochloride requirement) was dissolved in 50 cc. of water and treated with 50 cc. of 2N NaOH. The precipitated silver oxide was washed until the washings were neutral, and the silver oxide was then transferred with aid of the wash-bottle, to a solution of 9.66 gm. of arginine hydrochloride in 50 cc. of water. The mixture was stirred mechanically for ten minutes, and the silver chloride was filtered off. The filtrate was saturated with hydrogen sulfide, boiled, and the coagulated silver

sulfide filtered off. The filtrate from this operation was evaporated to dryness under reduced pressure on the water bath in a stream of carbon dioxide-free air. The residue was dissolved in 20 cc. of boiling water, and placed in a desiccator over sodium hydroxide. When the solution had cooled, the desiccator was evacuated. The yield was 7.84 gm. of arginine, melting at 228° (corr.) with decomposition. This was a 98 percent recovery from the hydrochloride. Recrystallization from 16 cc. of hot water and 40 cc. of freshly boiled absolute ethanol gave 96 percent recovery.

No difficulty was experienced in crystallizing arginine prepared from salmine or gelatin. One crystal of 3 cm. length was obtained by slow evaporation of the solvent during two weeks.

CITRULLINE

In Wada's classical paper on the chemistry of citrulline (15) the isolation of citrulline from watermelons, as well as a synthesis proving the structure of the amino acid were described. For the purposes of the present investigation ton quantities of watermelons would have been required to furnish enough citrulline. The synthesis described appeared to be equally impractical. The tryptic digestion of casein (16) presented the general objections of enzymatic processes, and other biological processes were even less attractive (17,18).

The utilization of the intermediates in the synthesis of ornithine described above appeared sufficiently promising to attempt. The steps involved were:

$NH_2(CH_2)_4COOH + HCNO$

$\downarrow$

$NH_2\text{-}CO\text{-}NH\text{-}(CH_2)_4COOH$

$\downarrow \quad + Br_2$

$NH_2\text{-}CO\text{-}NH\text{-}(CH_2)_3\text{-}CHBr\text{-}COOH$

$\downarrow \quad + NH_3$

$NH_2\text{-}CO\text{-}NH\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH$

Accordingly, a two-gram batch of delta carbamido valeric acid was synthesized by first rearranging cyclopentanone oxime (prepared for ornithine synthesis) with 80% H_2SO_4

to the lactam, and then hydrolysing with 15N H_2SO_4. The neutralized solution was evaporated with KCNO and a solid melting at 186° was isolated. This exhibited the correct properties for the carbamido acid. It was not found possible, however, to isolate a compound on bromination in carbon tetrachloride suspension of this carbamido acid. This is in accord with the observations of Schniepp and Marvel (19).

The next method of synthesis to be tried was the hydrolysis of arginine, according to the following reaction:

$$NH_2\text{-}C(NH)\text{-}NH\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH + HOH$$

$$\downarrow$$

$$NH_2\text{-}CO\text{-}NH\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH + NH_3$$

Because of the ready availability of arginine this appeared to be a promising practical process, but information in the literature was discouraging. Boon and Robson (20) have recorded an attempted isolation of citrulline from the alkaline hydrolysate of arginine. Ackermann had mentioned experiments of this sort under way or contemplated in 1931 (17) but no results have been reported.

Examination of the literature on the analogous hydrolysis of guanidine

$$NH_2\text{-}C(NH)\text{-}NH_2 + HOH \longrightarrow NH_2\text{-}CO\text{-}NH_2 + NH_3$$

revealed that although earlier workers found as products ammonia and carbon dioxide (21), Bell had obtained excellent yields of urea (22). Perusal of the reported conditions indicated the possibility that carbon dioxide and ammonia were obtained because guanidine was boiled with an excess of alkali. When only unimolar ratios of alkali to guanidine were employed the normal product was urea. Bell further found the same result using the free guanidine solution itself, which is alkaline. It seemed therefore that the often reported formation of ornithine by alkaline hydrolysis of arginine could be explained by the excess of alkali invariably employed.

Accordingly, hydrolysis of arginine with an NaOH solution in which the molar ratio of NaOH to arginine was 1:1, was attempted. The solution was boiled gently for 1 hour in an open Erlenmeyer flask. It was then cooled, neutralized, and treated with an excess of copper acetate solution. On standing, in the cold-room, the solution deposited crystals of a copper salt. Later an attempt was made to prepare a larger batch under reflux but no copper salt could be isolated. Assuming that the copper salt was copper citrullinate and recognizing that in the first run the solution had concentrated during boiling, it appeared likely that a low pOH as well as a proper $\frac{\text{NaOH}}{\text{arginine}}$ ratio was essential. Further experiments bore this out.

In order to follow hydrolyses quantitatively the gas

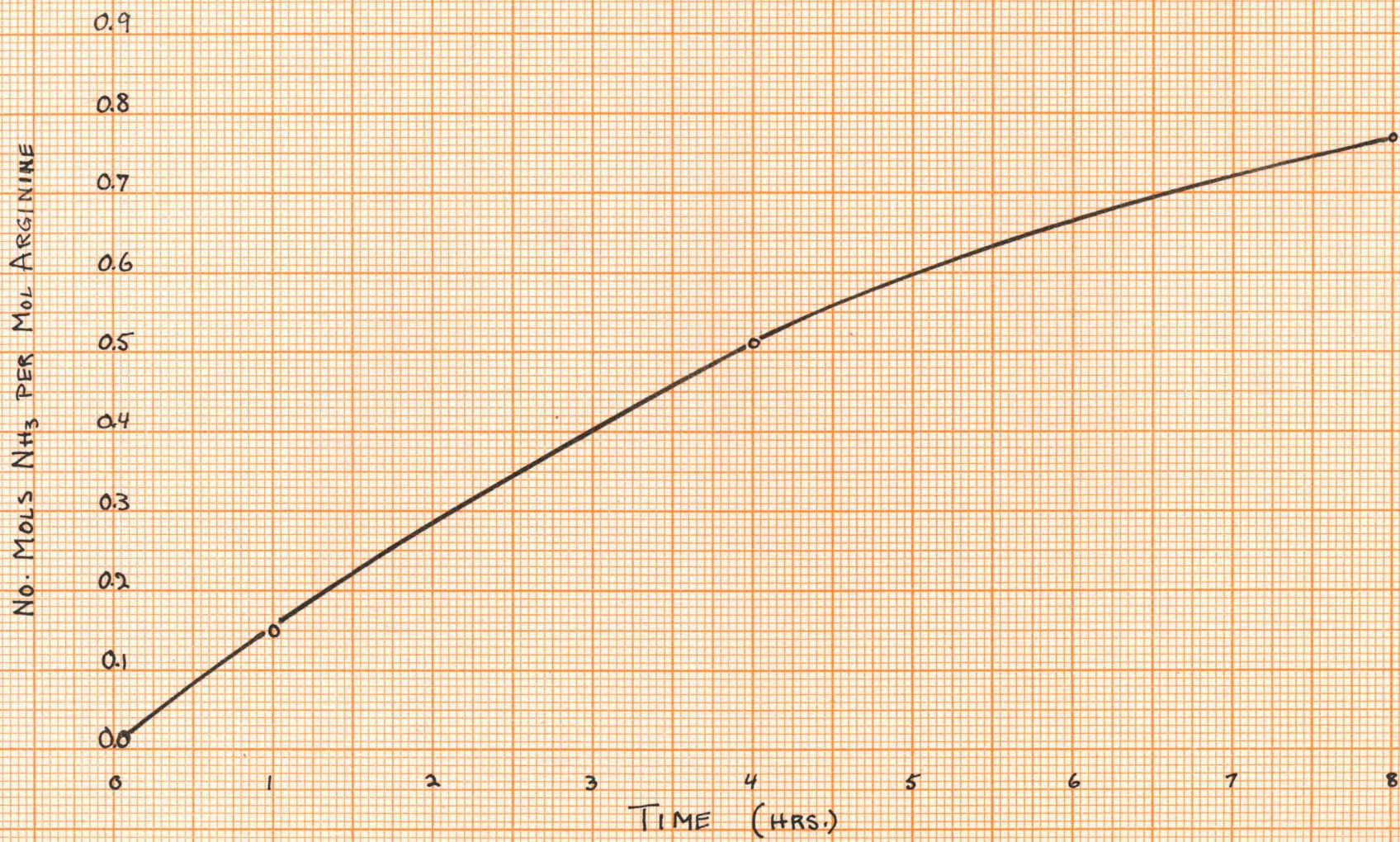
evolved was absorbed in a known volume of a standard HCl solution which was subsequently back titrated so that the amount of ammonia might be measured. If the reaction were not complicated by side-reactions one might expect the data to fit the typical equation for a first-order or possibly a second-order reaction. Neither of these was satisfied. The typical curve is illustrated in Graph I.

In another experiment a concentrated solution of free arginine was boiled 8 hours in a manner analogous to Bell's hydrolysis of guanidine alone. No ammonia was evolved. In yet another experiment an $\frac{18}{1}$ molar ratio of $\frac{NaOH}{arginine}$ was employed. No copper salt could be isolated when the reaction mixture was worked up in the usual manner described below, although theoretically sufficient ammonia had been given off. This lends strong support to the belief that the previously reported failure to isolate citrulline was due to an excess of hydrolysing alkali.

The conditions employed for preparing a large quantity of citrulline, were as follows: 198 gm. (0.938 mols) of arginine hydrochloride were brought carefully to boiling, under reflux, with 330 cc. of 5.68 N NaOH (1.876 mols, 0.938 mols for neutralization of hydrochloride). The flask was fitted with a condenser having an attached tube which led to slightly above the surface of a quantity of standard hydrochloric acid. The arginine solution, with

RATE OF HYDROLYSIS OF ARGININE TO CITROLLINE BY NaOH SOLN.

GRAPH I



glass beads was brought carefully to boiling, and refluxed 3 1/2 hours. At the end of this time, 0.64 mols of ammonia had been evolved. The solution was cooled, acidified with glacial acetic acid and evaporated under reduced pressure on the hot water bath to approximately 250 cc., 300 cc. of water was added, and the evaporation was repeated. The residue was treated with 1500 cc. of absolute ethanol, and the liquid poured off after separation was complete. The crude citrulline was washed with small quantities of alcohol and then dissolved in 800 cc. of water.

The solution was boiled for 30 minutes with 79 gm. of copper oxide, and the copper citrullinate and unchanged copper oxide were filtered off. In th smaller, pilot runs, no copper citrullinate crystallized at this stage, and it was possible to filter off copper oxide alone. The filtrate was evaporated down over a steam bath, and successive crops of copper citrullinate were removed by filtration. Four such crops were combined with the residue from the original filtration and the entire batch was washed thoroughly with water until the washings were colorless. A sample recrystallized from water decomposed at 257-258° (corrected). The decomposition point of the copper salt is highly reproducible, whereas for the free citrulline values from 202 to 226° are recorded in the literature, and values in the interval 200-218° were found in this work. The copper salts were suspended in one liter of water, which was then saturated with hydrogen sulfide.

The copper sulfide was coagulated by boiling, filtered hot, and the filtrate was cleared with norit. The solution was evaporated under reduced pressure on a hot water bath until crystals began to appear and it was then treated with several volumes of absolute ethanol. The crystals which separated were dried in vacuo over phosphorus pentoxide. The yield was 65 gm. of white citrulline.

A sample was recrystallized from water and alcohol.

$C_6H_{13}N_3O_3$. Calculated. Amino N 7.99

Found, " " 7.94, 7.89 (microformol)

When examined in aqueous solution, and in dilute hydrochloric acid, the citrulline showed no optical activity.

After a second recrystallisation from water and ethanol the citrulline was used for heat capacity determinations.

Sample (a) Some of the citrulline from the heat capacity runs was thrice crystallised from concentrated aqueous solution by the addition of ethanol.

Sample (b) Two batches of citrulline residue from recrystallisations were each crystallised twice from water and combined.

Sample (c) This was a sample prepared in the same manner as (a). Because alcohol treatment gave poor samples of high heat values, this sample was not burned.

Sample (d) A new batch of citrulline was prepared from gelatine via arginine flavianate, benzylidene arginine, and arginine hydrohloride as previously described. Since the two previous samples showed an appreciable ash content, this batch was treated with H_2S twice in the belief that any cupric or cuprous material which might have remained behind from the first H_2S treatment, would be removed. Whether or not this explanation were correct the ash residue from combustions of sample (d) were unweighable. The citrulline was then crystallized twice from water and ethanol and twice from water alone.

Sample (e) This was citrulline from the residues of sample (d). As in the case of the non-homogenous sample (b), this material was of low purity since it was reclaimed citrulline and gave a combustion value in poor agreement with samples which were believed to be of higher purity.

Sample (f) The remainder of sample (a) was again treated with H_2S in the manner successfully applied to (d) and twice crystallized from water.

Samples (d) and (f) were probably the best of the various preparations.

Formol amino nitrogen analysis of the original citrulline used for heat capacity determination gave 7.92 percent as against the theoretical of 7.99.

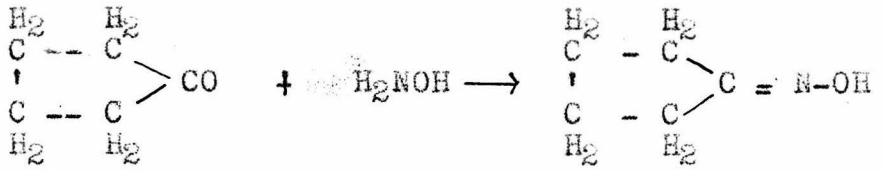
ORNITHINE

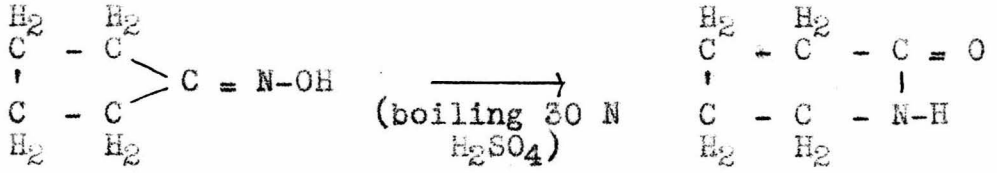
Vickery and Cook (23) obtained crystalline d-ornithine by enzymatic decomposition of arginine. The product decomposed at 140°. Since the material first obtained was highly contaminated and the process did not lend itself to the preparation of 30 gram lots, a better method was sought. These authors recorded the difficulties of handling ornithine because:

(1) It is highly hygroscopic.
(2) It is sufficiently basic to absorb atmospheric carbon dioxide.
(3) When stored in the dark in sealed vessels, it decomposed slowly.

The above properties were checked in this work with dl-ornithine.

The advisability of preparing ornithine by direct isolation as against a more synthetic method has been discussed above. A synthesis appeared to be preferable. The author had already elsewhere developed a synthesis of ornithine by the following steps:

(a) 

(b) 

(c) H_2O (boiling 15 N H_2SO_4)

↓

(d) $NH_2(CH_2)_4 - COOH$ + C_6H_5COCl

↓

(e) $C_6H_5CO-NH(CH_2)_4-COOH$ + Br_2

↓

(f) $C_6H_5-CO-NH-(CH_2)_3-CHBr-COOH$ + NH_3

↓

(g) $C_6H_5-CO-NH-(CH_2)_3-HCNH_2-COOH$

In spite of generally excellent yields, the combined step (f) and (g) had given yields of about 20 percent. The synthesis was again checked in this laboratory with variations of molar ratios, times of heating, etc.. None of these modifications resulted in an improved yield, so

it was felt that this synthesis would not furnish enough material to resolve.

Since it is known that an undesirable by-product appeared in the bromination process described above and that this involves the imino hydrogen (24)

$$C_6H_5CO-\overset{*}{N}H-(CH_2)_3-CH\overset{*}{N}H_2-COOH$$

it was felt that it might be possible to use the corresponding benzylidene derivative

$$C_6H_5-C=N-(CH_2)_3-HC\overset{*}{N}H_2-COOH$$

since there were here no reactive hydrogens on the N. An alkaline solution of $NH_2(CH_2)_4-COOH$ was therefore treated with a theoretical excess of benzaldehyde in the usual fashion (25) but no crystalline material could be isolated when the mixture was allowed to stand in the cold overnight.

What for long had been the most popular method for preparation of ornithine salts (26), alkaline hydrolysis of arginine, was also tried. This method was abandoned since it gave only a small yield of a partially racemized product.

More hope was felt at this stage for the resolution of the benzoyl derivative of ornithine. It seemed likely that acid hydrolysis of the resolved ornithuric acid (dibenzoylornithine) might yield a product racemized to a

small enough extent that it could be readily purified by recrystallisation.

Accordingly, several batches of ornithuric acid were prepared by the method of Boon and Robson (20) and resolved by the procedure of Sorenson (27). Inasmuch as resolution of amino acids is often simpler through the formyl derivatives than the benzoyl, an attempt was made to prepare a crystalline formyl derivative. A sample of ornithine was refluxed with 99% formic acid (28). The product obtained was dissolved in water and precipitated with acetone and ether, but after several days in the ice-box, the mixture failed to yield crystals.

Since Berg had successfully used d-camforic acid in the direct resolution of lysine (29), it seemed likely that ornithine, the next lower homolog of lysine, could be similarly resolved, possibly with a more satisfactory yield. Camforic acid was prepared by oxidation of camfor (30) and it was possible to form what was probably d-ornithine d-camforate. The compound isolated had a melting range of 229-31° (d-camforic acid melts at 77°, ornithine decomposes at 146-7°, d-lysine d-camforate melts at 239-40° (29). Here, as in the case of lysine, it was necessary to recrystallise many times to obtain a product with a constant high melting point. This did not furnish a sufficiently convenient resolution.

Sorenson's resolution of ornithuric acid prepared by the process of Boon and Robson was therefore employed. The method is schematically:

$NH_2(C{=}NH)\text{-}NH(CH_2)_3\text{-}CHNH_2\text{-}COOH + NaOH$

↓

$NH_2\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH + 2C_6H_5COCl \quad (NaOH)$

↓

dl $C_6H_5\text{-}CO\text{-}NH\text{-}(CH_2)_3\text{-}C_6H_5CONHCH\text{-}COOH$

l-brucine dl-ornithuric acid

↓

l-brucine d-ornithurate.

l-brucine d-ornithurate + NaOH (cold)

↓

l-brucine Na d-ornithurate

$d\text{-}C_6H_5\text{-}CO\text{-}NH\text{-}(CH_2)_3\text{-}C_6H_5CONHCH\text{-}COOH + HCl \quad (conc.)$

↓

$d\text{-}HCl\cdot NH_2\text{-}(CH_2)_3\text{-}CHNH_2\text{-}COOH\cdot HCl$

Two preparations of d-ornithine dihydrochloride having specific rotations of -5.6 and -9.8° respectively were made. Since the best d-ornithine dihydrochloride rotation was -16.8°, racemization evidently had accompanied the hydrolysis of ornithuric acid. Recrystallisation of free d-ornithine appeared to be a more promising means of purification than recrystallisation of the hydrochloride. Free d-ornithine prepared from the hydrochloride by the use of silver oxide, was recrystallised. The specific rotation read -11.0° as against the best recorded value of -11.5°. This line of attack, however, proved not to be the solution it at first seemed since on recrystallisation

d-ornithine racemized. For example, a material showing an initial specific rotation of -6.4° showed -2.4° after recrystallisation with noriting. Noriting hot solutions would be essential, after the heat capacity runs, in order to decolorize ornithine for combustion samples. It seemed likely at this stage of the ornithine problem that it would be essential to prepare and employ dl-ormithine.

dl-Ornithine was therefore made. The present melting point of 147-8°, with decomposition, is the first given. Vickery and Cook (23) reported the melting point of d-ornithine as 140°. Since the details of the preparation are not to be found in the literature they are presented here:

Eight hundred eighty-two gm. of arginine flavianate prepared by the method previously mentioned (8,9) were heated with 1700 cc. of concentrated HCl on a boiling water bath for two hours. The contents of the flask were occasionally stirred. The mixture was allowed to cool, and the flavianic acid was filtered off, washed with 10 percent hydrochloric acid, and the combined filtrate and washings were evaporated under reduced pressure on a hot water bath. Following additions of one liter portions of water, the evaporation was thrice repeated. The final residue was dissolved in one liter of water.

After standing, the liquid was filtered, diluted to 2.5 liters and cleared by boiling with 10 gm. of norite for 10 minutes. The filtrate was neutralized to litmus with approximately 110 cc. of 50 percent NaOH solution. One kg. of solid NaOH was now added and the liquid was brought carefully to boiling under reflux. After 12 hr. of refluxing, one liter of water was added and the refluxing was continued for 6 hrs.

The cooled liquid was partly neutralized with 500 cc. of concentrated HCl. This mixture was kept below 10°, with mechanical stirring in an ice-bath, and 990 cc. of benzoyl chloride were added dropwise. It was necessary to dilute to 11.5 liters with water during the benzoylation. The mixture was next acidified to Congo Red with 700 cc. of concentrated HCl.

The resultant precipitate was filtered with suction and washed well with one liter of water and three one liter portions of absolute ethanol. The residue was recrystallised from 10 liters of boiling 40 volume percent ethanol. The dried product weighed 419 gm. and a sample melted at 186°.

One hundred twenty-nine gm. of this ornithuric acid was refluxed for 19 hours with 2 liters of concentrated HCl. The benzoic acid formed by this treatment was filtered from the cooled reaction mixture and was washed with 10 percent HCl. The combined liquids were evaporated under

reduced pressure, treated with a few cc. of concentrated HCl, and set in vacuo over H_2SO_4. The mass dried and crystallised in three days, was washed with 300 cc. absolute ethanol, and dried again in vacuo over sulfuric acid to give 70 gm. of white ornithine dihydrochloride.

In order to decompose the hydrochloride 70.0 gm. was treated with the Ag_2O prepared from 118.3 gm. (1 percent excess) of $AgNO_3$ and 1 mol. of NaOH in water, stirred mechanically for 15 minutes, filtered, and saturated with H_2S.

The liquid was centrifuged from the Ag_2S, evaporated under reduced pressure to about 200 cc., boiled 2 minutes with ½ gm. of norite previously extracted with boiling water, evaporated under reduced pressure in a stream of CO_2-free air, and transferred to a desiccator over NaOH and H_2SO_4. The desiccator was evacuated when cool.

When the solution had evaporated to a thick syrup containing crystals it was treated with 400 cc. of aldehyde-free absolute ethanol (31) and 300 cc. of aldehyde-free ether (32) and set in a CO_2-free space. The crystals were filtered off after three days, dissolved in 30 cc. of hot water, and again crystallised in the same fashion. After drying, the ornithine was found to consist of prisms melting at 146-8° with decomposition.

THE HEAT CAPACITIES AND ENTROPIES OF THE COMPOUNDS

Method

For the evaluation of the entropy of a substance the heat capacity is determined as a function of temperature from liquid air temperatures to the temperature at which the entropy content is desired. Assuming the entropy of a pure crystalline substance at the absolute zero to be, according to the third law of thermodynamics, zero, the entropy at any other temperature, T_1, is then

$$S_T = \int_0^{T_1} C_p \, d \ln T + \frac{\Delta H}{T}$$

$\frac{\Delta H}{T}$ represents the change in heat content at the temperature at which a transition occurs.

One experimentally determines the heat capacity curve from the temperature attainable with liquid air and then extrapolates the curve to 0°K by the method of Kelley, Parks, and Huffman (33).

The method and apparatus used here were essentially those described by Parks (34). The apparatus consisted of a cylindrical copper or gold calorimeter wound with a heating coil, and fitted with a single junction copper constantan thermocouple for temperature measurements. The calorimeter was filled with pellets of the substance to be studied, interspersed with perforated metallic disks to facilitate heat conduction. In the case of ornithine, the material was soaked in anhydrous petroleum ether before pelletting.

After the pellets were packed into the open calorimeter, one end being removable, they were dried to constant weight. At this stage the removable end was soldered into place. In order to minimize radiation, the calorimeter was tightly enclosed in a sheath of thin platinum foil. The sheathed calorimeter was now suspended by a silk thread from the top of a heavy copper shield which could be maintained at any desired temperature above that of the bath. The shield was then placed in a can from which it was insulated by hardwood wedges. This can was sealed to a glass system which was evacuated first by an oil pump and finally by a charcoal trap in liquid air. The can and its contents were cooled by immersion in a bath of liquid air, solid CO_2 and alcohol mush, or ice water.

Temperature measurements were made on a White double potentiometer in conjunction with a suitable galvanometer and connected to the calorimetric system by appropriate leads. The energy input was calculated from measurements of current, voltage, and time of energy input with the aid of a reliable stop watch.

From the equation

$$Cp = \frac{dQ}{dT} \text{ calories per gram per degree}$$

the heat capacities were calculated in the range 90° - 300° K. To obtain the heat capacity of the substance investigated the heat capacities of the metals in the set-up were subtracted from the total.

The extrapolation formula used was

$$Cp = (A-BT)Cp^{o}$$

in which Cp^{o} is the molal specific heat of the standard substance. For straight-chain compounds such as citrulline and ornithine, A and B are evaluated as follows:

$$B = \frac{Cp_{120^{o}} - 1.2137\ Cp_{90^{o}}}{351},$$

$$A = \frac{Cp_{120^{o}} - 1404\ B}{11.70}.$$

The results of the heat capacity determinations for ornithine are given in Table I, those for citrulline in Table II, and the entropies in Table III. The values are presented as specific heats, i.e., heat capacities per gram of substance, or calories per gram per degree.

Table I

Specific Heats of Ornithine

Temp ^{o}K	Calories/gram/degree
88.6	.1257
94.6	.1298
101.1	.1364
109.5	.1464
126.0	.1653
144.1	.1821
156.3	.1933
178.6	.2131
193.7	.2276
209.1	.2423
223.8	.2563
237.0	.2681
263.8	.2951
277.0	.3087
277.1	.3096
283.0	.3175
288.5	.3274
290.8	.3324
298.0	.3441

GRAPH II

Specific Heats of Ornithine

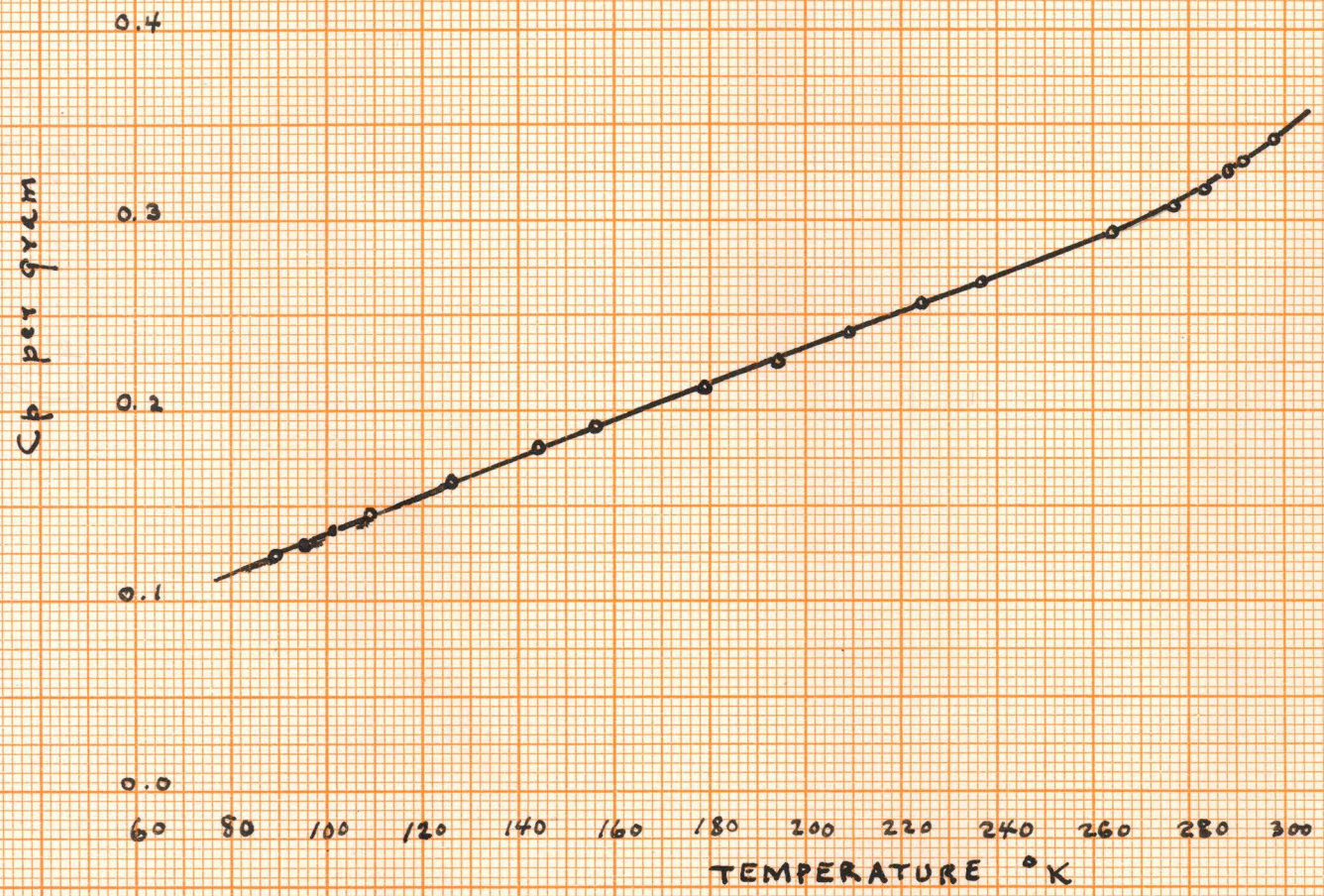


Table II

Specific Heats of Citrulline

Temp °K	Calories/gram/degree
89.4	.1261
95.7	.1324
101.7	.1383
110.1	.1463
125.5	.1608
140.7	.1740
157.3	.1889
177.3	.2069
194.5	.2222
207.9	.2335
223.5	.2483
239.4	.2622
264.8	.2844
277.5	.2957
283.9	.3022
290.5	.3076
300.8	.3176

GRAPH III

SPECIFIC HEATS OF CITRULLINE

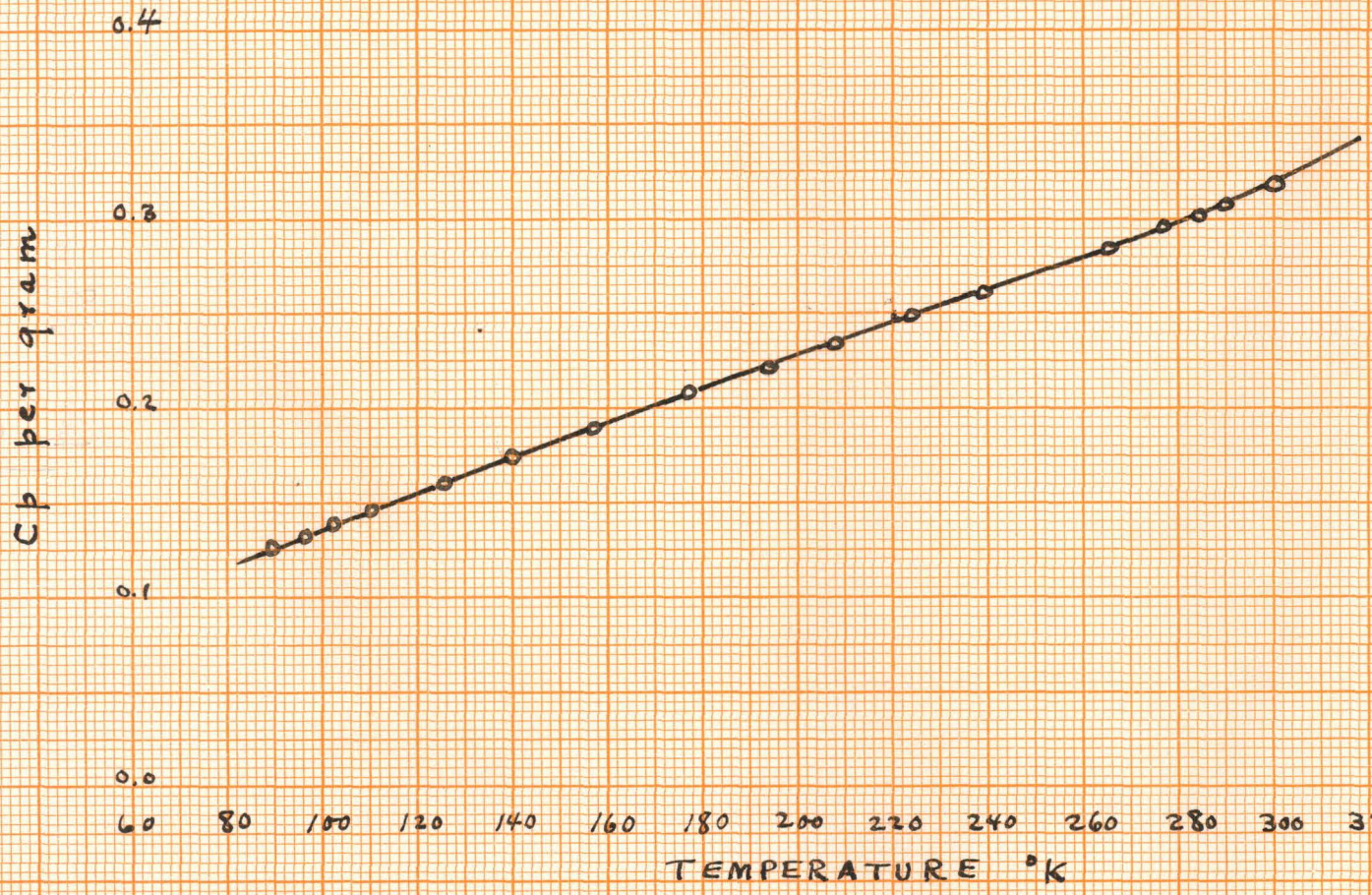


Table III

Entropies of the Compounds
(E.U. per mol)

Substance	S 90°	S 90°-298.1°	S 298.1°
Ornithine	13.16	33.05	46.21
Citrulline	18.15	42.57	60.72

Heats of Combustion

The apparatus and method have previously been described by Huffman, et al. (35,36,37). The combustions occurred in a Parr bomb placed in a stirred calorimeter surrounded by a jacket maintained at a temperature constant to about 0.01°. An amount of substance estimated to cause a temperature rise of the calorimeter of about 2.00° was weighed out and compressed into a pellet. The pellet was then dried to constant weight in a weighing bottle in a desiccator containing phosforic anhydride, in an oven at 100°, or in a vacuum oven at a lower temperature. The mass was then accurately determined on a micro-balance to 0.01 milligram, and placed in a platinum crucible.

The crucible containing the pellet was set in a ring suspended from the top of the bomb. A platinum wire extended circularly above this ring. This wire could be brought to incandescence electrically and contained a loop to which a paper fuse was connected by a hook in the fuse. This fuse was placed so as to fall on the pellet when it ignited and thus initiate combustion of the pellet, which was generally less readily combustible than the paper. Fuses were kept at constant humidity and weighed just before use. The heat of combustion of the paper at constant humidity had previously been accurately determined.

One cc. of water was added to the bomb to absorb gaseous combustion products, and the lid was placed in position and locked with a threaded nut. The bomb was then evacuated to a pressure of a few millimeters of mercury through a valve in the

cover and filled with oxygen to 30.0 atmospheres of pressure.

The calorimeter was filled with water to a tared value which was the same as used in determining the heat capacity of the assembled calorimeter. The bomb was placed in the calorimeter and electrical connections for firing were made.

The temperature of the calorimeter was followed with a platinum resistance thermometer in conjunction with a suitable galvanometer connected with a Mueller bridge. As the calorimeter water reached each of a series of predetermined temperatures, the time was recorded on a chronograph. At a higher predetermined temperature the sample was ignited by bringing the platinum wire in the bomb to incandescence with an automatically controlled amount of electrical energy. This energy always contributed 1.4 ± 0.1 calories to the heat evolution.

As the sample ignited, heat was given off to the calorimeter, and the temperature rose rapidly. The times at which predetermined temperatures were attained were recorded as before. At near the end of the rise, each of the smallest registrable temperature increments of .001° was recorded for many minutes as the calorimeter cooled toward the lower temperature of the jacket.

With the above data, the temperature rise due to combustion could be corrected for heat transfer from and to

the jacket in the warming and cooling periods respectively.

The bomb was next taken apart and the interior washed thoroughly. The washings were titrated for HNO_3 formed. The principal nitrogenous product of combustion of nitrogen-containing samples was N_2 but a few percent were always oxidised to HNO_3. The heat of combustion value was corrected for the heat of formation of HNO_3 found as well as for the paper fuse and electrical energy input.

A minimum ash content of all samples was determined by heating the weighed crucible to red heat after the combustion and weighing the crucible again. This served as an index of non-volatile inorganic impurity.

The citrulline pellets were hygroscopic, necessitating drying at 60° in vacuo and weighing of the pellets by taking the differences in weight of a weighing bottle containing a pellet, and when empty. The precision of the citrulline values is not as high as for fumaric and maleic acids, described in the section on Thermal Data for Other Biochemicals, in each of which cases, variations were no greater than 1.5 calories per gram, or 0.06 percent.

One pellet of sample (f) was left in the bomb for 30 hours at atmospheric pressure. The combustion value for this pellet was 8 calories lower than for other determinations on the same sample. A probable explanation is hydration during standing under such conditions. Since the standard combustion occurs within less than an hour after introduction of the pellet into the bomb and the pressure within the bomb is at the

high value of 30 atmospheres, the lowering in heat of combustion due to hydration during each of the standard combustions would reasonably be a very small fraction of the lowering in the special case above. The absolute error resulting during combustion in the normal manner from this factor then appears to be quite small, probably less than one percent of the lowering in the special case. Such an error should be less than 0.1 calorie, which is negligible compared to the deviations in the combustions.

During the period in which combustions of citrulline were made, the heat capacity of the calorimeter was frequently determined by combustion of Bureau of Standards benzoic acid. Values between 3152.3 and 3155.2 calories per degree were obtained. The extreme deviation was thus 0.09 percent. The deviation of values obtained by experts such as those in the Bureau of Standards is $\pm$ 0.03 percent (37).

The heats of combustion, as reported in table IV, were corrected to the standard states of the elements by the Washburn correction modified for nitrogenous substances (36). The values given in table IV are for a reaction occurring isothermally at 25°C. The precision of the values recorded is .11 percent and the accuracy presumably has a numerical value slightly greater. The values recorded are for samples (d) and (f). The total number of combustions was fourteen.

Table IV

Heats of Combustion of Citrulline

(Calories per gram in vacuo at 25°C.)

Sample	$-\Delta U_B$
d	4584.5
d	4582.4
f	4587.4
f	4587.4
mean	4585.4

A number of combustions were run on three samples of ornithine. These gave divergent values. In three cases, increases in heats of combustion were noticed on extended drying of companion samples, in one case a decrease. There are probably a number of reasons for these discordant values; tenaciously held moisture, tightly held organic solvent, organic impurity, instability to warm drying, etc. Submission of two samples to C, H, and N analysis revealed neither the requisite purity nor reasonable explanation of the combustion values obtained (thanks are here expressed to Doctors Haagen Smit and Prater for these and other analyses). The fact that the C content and H content were equally low (approximately two percent in one case) would not agree with the postulate of the presence of moisture, for instance. Because of the evidence of insufficient purity for the determination of an accurate heat of combustion, the values obtained were not used, and the free energy of formation of ornithine was arrived at by another route, as described in the section on Derived Data.

Solubilities

Solubilities were determined by shaking the solids with water in stoppered ⊥-shaped solubility tubes in a water thermostat at 25.00 ± 0.03 °C. for at least 48 hours. Values were determined at longer intervals in cases such as citrulline, in which the equilibrium between solution and solid phase was established more slowly. Samples were removed from the tubes by suction through a bent glass tube containing a cotton plug to filter out solid particles. The solution was drawn into a tared weighing bottle and the amount of solution determined by difference in weight. The liquid was then concentrated in a vacuum desiccator over phosforic anhydride, and the residue finally dried at 60-65° C. in a vacuum oven. Ornithine, however, was dried only in the desiccator since samples of it decomposed at elevated temperatures. The samples were considered dry when their weights were constant to about 0.2 milligram.

For each of the substances, solubility determinations were first run with a mixture of water and excess solid at 25°, and later with an initially supersaturated solution obtained by warming the first mixture until clear. Samples were removed until consecutive solubility values checked within the experimental error, and until the

values for the initial mixture and those from the warmed mixture similarly agreed. The solubilities of arginine exhibited an extreme deviation of 0.6 percent, those of citrulline 3 percent, while for ornithine the spread was 4 percent.

The solubilities are presented in Tables V to VIII inclusive.

Table V

Solubility of d-Arginine in Water at 25.00°

Weight of Solution grams	Weight of Solid grams	Mols Anhydrous Arginine / Kg. water
1.0988	0.2401	1.606
0.6920	0.1505	1.596
1.3500	0.2944	1.602
1.4127	0.3083	1.603
	Av.	1.602

Table VI

Solubility of Citrulline in Water at 25.00°

Weight of Solution grams	Weight of Solid grams	Mols Anhydrous Citrulline / Kg. water
1.0021	0.1535	1.033
1.2565	0.1878	1.003
0.4471	0.0677	1.019
0.3020	0.0462	1.031
1.3709	0.2082	1.022
	Av.	1.022

Table VII

Solubility of dl Ornithine in Water at 25.00°

Weight of Solution grams	Weight of Solid grams	Mols Anhydrous Ornithine / Kg. water
0.1938	0.1405	19.9
0.0959	0.0695	19.9
0.0439	0.0315	19.2
0.1072	0.0768	19.1
	Av.	19.5

Table VIII

Solubilities of the Compounds at 25° C.

Substance	Solubility Mols/kilogram of water
d-Arginine	1.602
Citrulline	1.022
Ornithine	19.5

Dissociation Constantsof Citrulline

Since the dissociation constants of arginine and ornithine had been previously reported (38,39) it was necessary to obtain the dissociation constants for citrulline only. This was done by electrometric titration.

The titration was conducted in a 30 cc. beaker at 25°. A measured quantity of citrulline solution of known concentration was titrated with 0.1112 N NaOH solution or 0.0706 N HCl solution from a buret which could be read to 0.01 cc.± 0.003 cc. The quinhydrone electrode was freshly made and calibrated against a standard potassium acid phthallate buffer solution having a pH of 3.97.

After definite volumes of NaOH or HCl solution were added, and the solution was stirred, the pH was measured with a Beckman pH meter until constant on continued stirring.

pK_A was calculated from the equation:

$$pK_A - pH = \log \frac{A - (\overset{+}{H})}{C - (\overset{+}{H}) - A}$$

where A = concentration of added acid in mols per liter and C = concentration of citrulline in all its forms in mols per liter

pK_B was similarly calculated thus:

$$pK_B - pOH = \log \frac{B - (\bar{OH})}{C - (\bar{OH}) - B}$$

The values are presented in Tables IX and X.

Table IX

Titration of Citrulline for pK_B

10.00 cc. 0.04693 N citrulline

titrated against 0.1112 N NaOH at 25°

cc. NaOH	pH	pK_B
0.00	5.69	
0.10	7.38	5.05
0.20	7.64	5.06
0.30	7.82	5.06
1.00	8.44	5.05
1.25	8.56	5.06
1.75	8.82	5.03
3.00	9.27	5.12
	mean	5.06

Table X

Titration of Citrulline for pK_A

10.00 cc. 0.04693 M citrulline

titrated against 0.0706 N HCl at 25°

cc.HCl	pH	pK_A
0.45	3.55	2.37
0.55	3.47	2.37
0.75	3.30	2.35
1.15	3.09	2.35
1.75	2.86	2.33
3.00	2.55	2.32
4.00	2.39	2.33
5.00	2.24	2.36
	Mean	2.35

Thermodynamic Activities

The activities, or effective concentrations, of the substances in aqueous solution were calculated from the vapor pressures of various concentrations of aqueous solutions at 25°. These in turn were measured by a semimicro modification of the "isopiestic" method developed by Robinson and Sinclair (40).

Platinum boats 15 mm. long, 15 mm. wide, and 5 mm. high were made by folding platinum foil. Four of these were placed on a silver-plated copper block 50 mm. in diameter and 25 mm. thick. In two boats placed diagonally were put samples of the solution under investigation, with concentrations a few percent different. In the other two boats were similarly placed unequal concentrations of solutions of a standard material such as KCl or urea.

Since a number of desiccators were required for these determinations , they were made from sturdy 500 cc. bottles. The bottoms of the bottles were cleanly cut off with a hot wire and ground to a thick glass plate. The mouth of each bottle was fitted with a stopcock in a rubber stopper. These served satisfactorily when the ground surfaces were greased.

The set of solutions was placed in one of the desiccators

which was evacuated and placed in a 25° air thermostat. After many hours, the desiccator was opened and the boats immediately weighed. When properly standardized these weighings could be made accurate to 0.2 milligram for solutions of about 200 milligrams.

Solutions about 1 molal with an initial concentration difference of 3 percent, for instance, would reach equilibrium within 24 hours. The less concentrated the solution, the more slowly, in general was equilibrium attained. Since the isopiestic method can be applied with high accuracy it is to be preferred to the freezing- point method for determination of activity (41) when the substance has moderate solubility. It also avoids the errors of extrapolations from freezing temperatures to 25°.

When the equilibrium concentrations of the unknown against the standard were evaluated for a range of approximately 0.5 molal to the saturated solution, the vapor-pressure lowering was then calculated for the found concentration of standard. This was then the identical value for the unknown solution. The worth of these figures could then be evaluated by inspection, when concentrations of unknown were plotted against vapor-pressures and a curve drawn through the points.

It was possible then to evaluate the activity of the solute by the h/m function of Lewis and Randall (42). This function gives a graph which greatly magnifies any aberrations in the vapor pressure curve. Using the h/m function it is now possible to evaluate activity elegantly by selecting points from the smoothed vapor pressure-concentration curve. With the exception of extrapolated points, the actual experimental values proved to be accurate enough to use directly.

The data obtained are presented in Tables XI to XIII inclusive.

Table XI

Activity of Citrulline at 25.0°

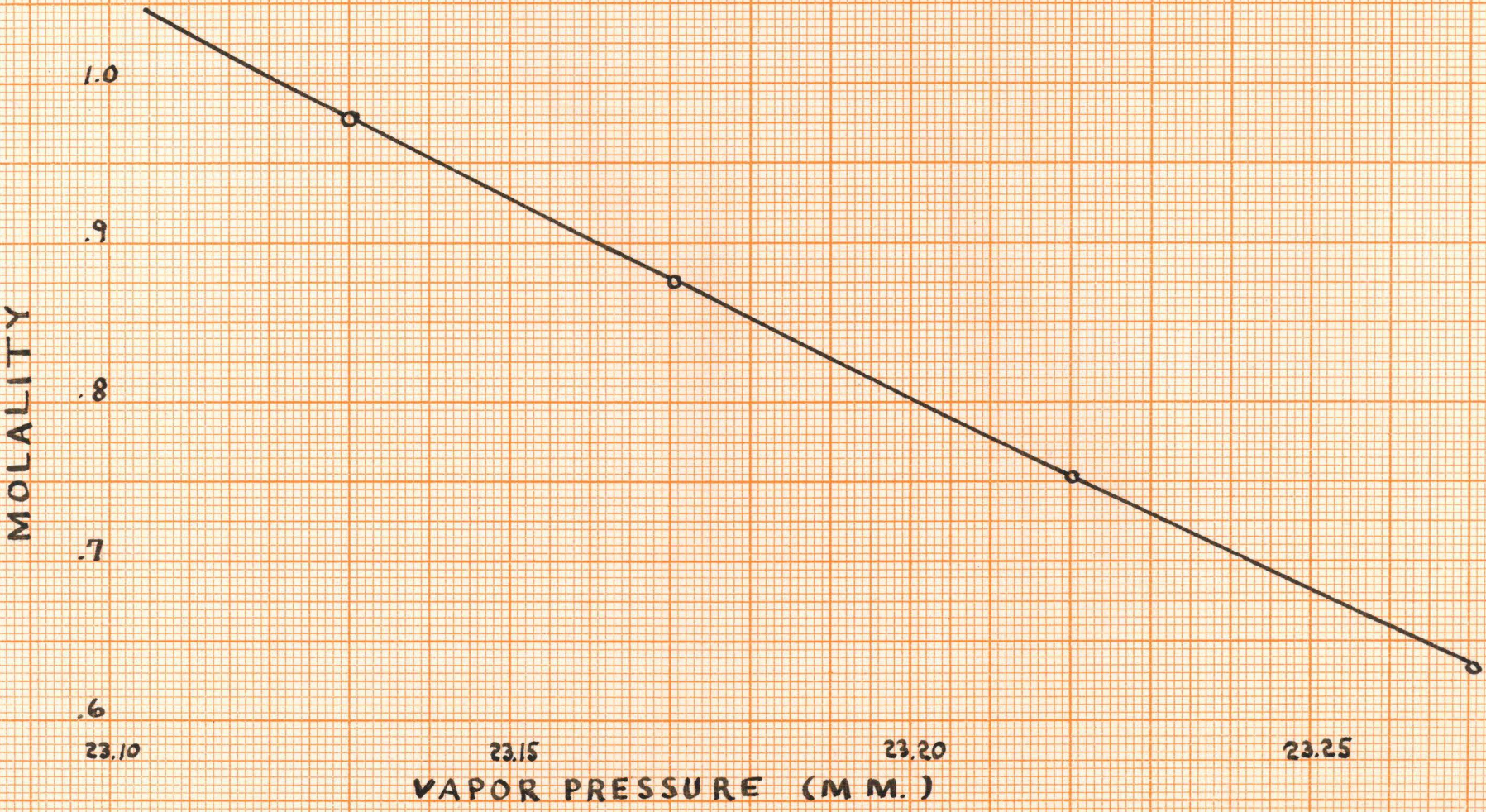
Molality of Citrulline Solution	Isotonic Molality of KCl Solution	Vapor Pressure
.6376	.3273	23.27 mm.
.7546	.3962	23.22
.8761	.4515	23.17
.9783	.5148	23.13

Activity of Citrulline in Saturated Solution: .8608

Activity Coefficient: .84

GRAPH IV

VAPOR PRESSURES OF AQUEOUS CITRULLINE SOLUTIONS



GRAPH V

ACTIVITY OF CITRULLINE SOLUTIONS

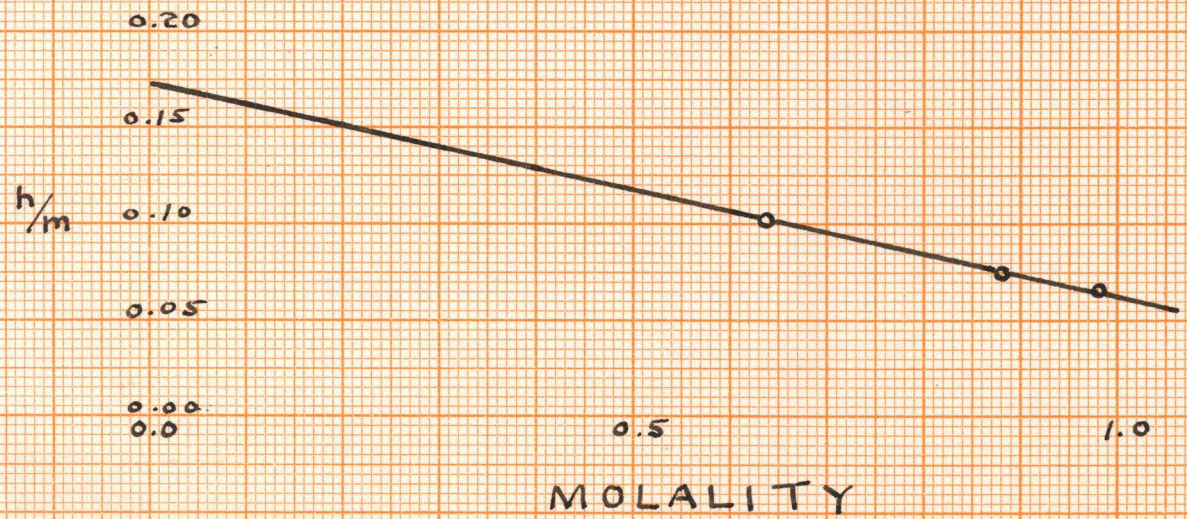


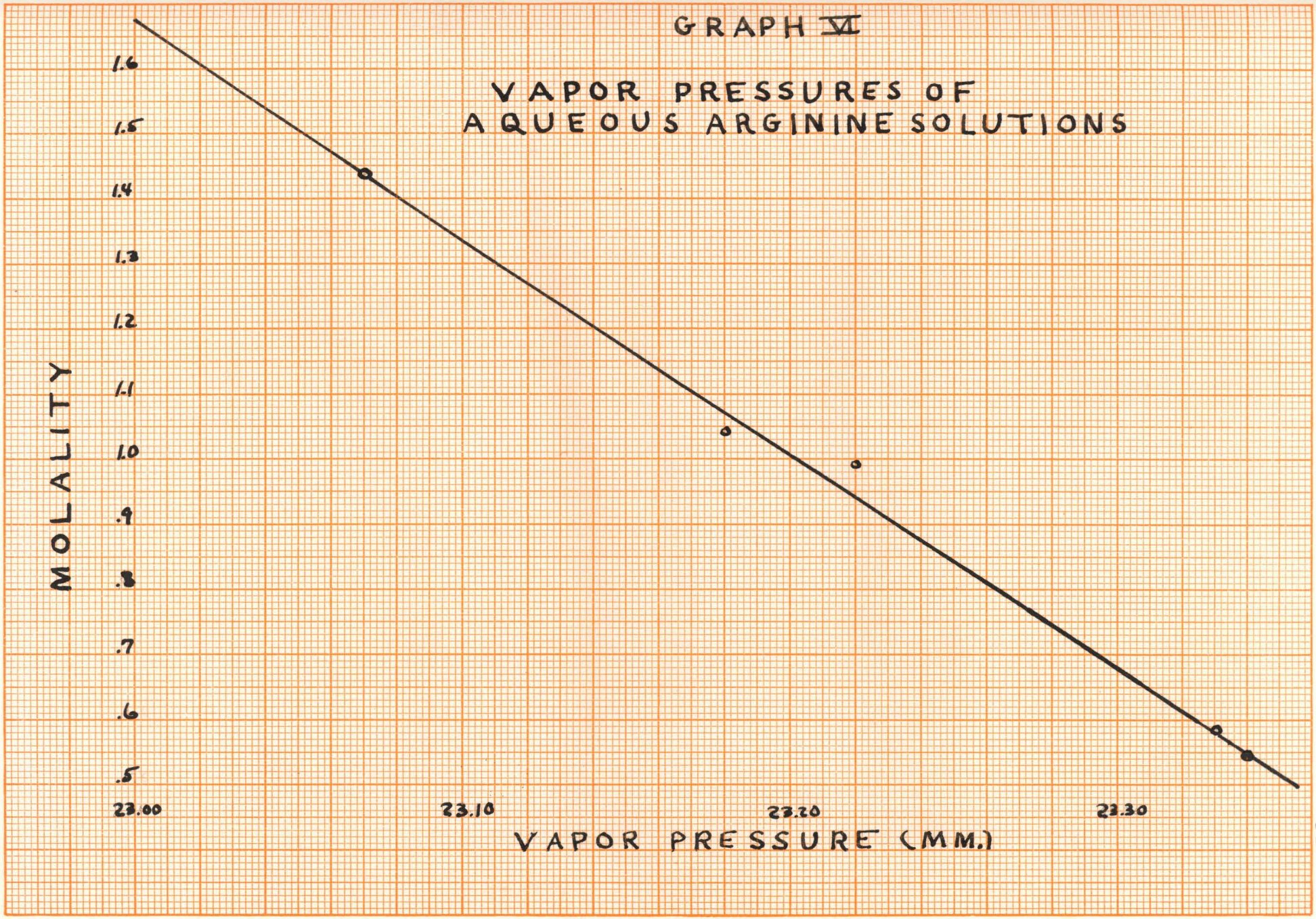
Table XII

Activity of Arginine at 25.0° C.

Molality of Arginine Solution	Isotonic Molality of KCl Solution	Vapor Pressure
.5479	.2354	23.34 mm.
.5841	.2507	23.33
.9934	.3942	23.22
1.043	.4530	23.18
1.438	.5964	23.07

Activity of Arginine in Saturated Solution: .5878

Activity Coefficient: .36


GRAPH VI
VAPOR PRESSURES OF
AQUEOUS ARGININE SOLUTIONS
MOLALITY
1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
23.00
23.10
23.20
23.30
VAPOR PRESSURE (MM.)

GRAPH VII

ACTIVITY OF ARGININE SOLUTIONS

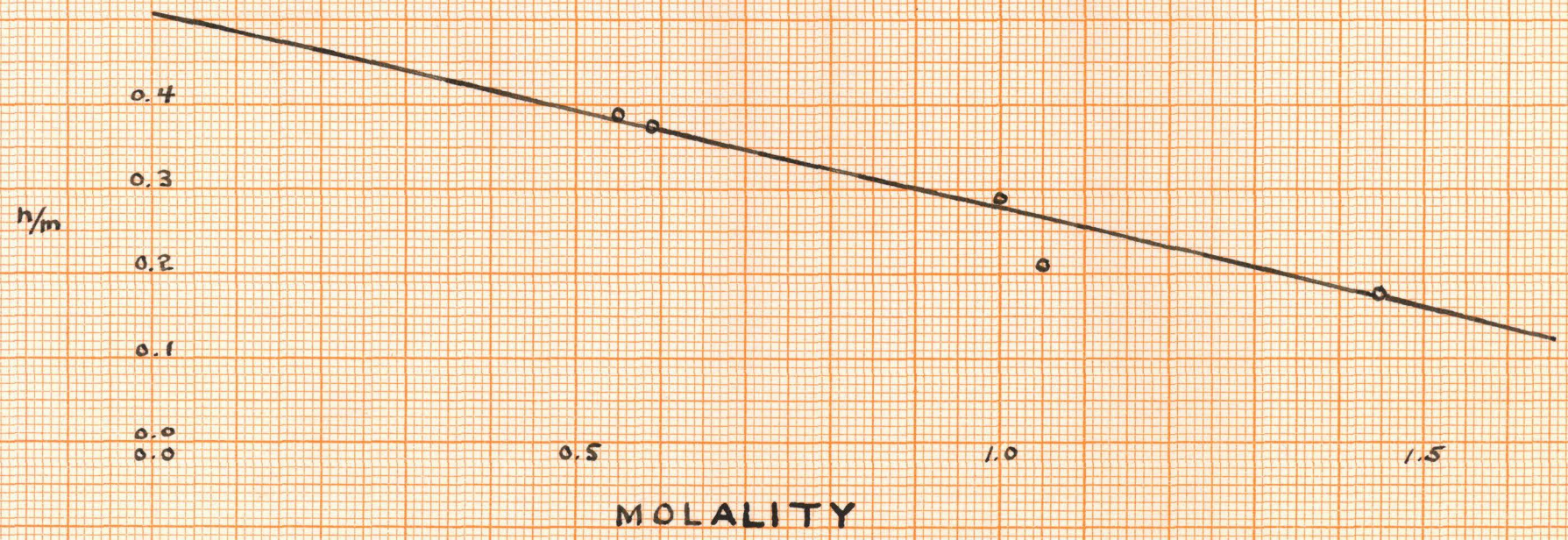


Table XIII

Activity of Ornithine at 25.0° C.

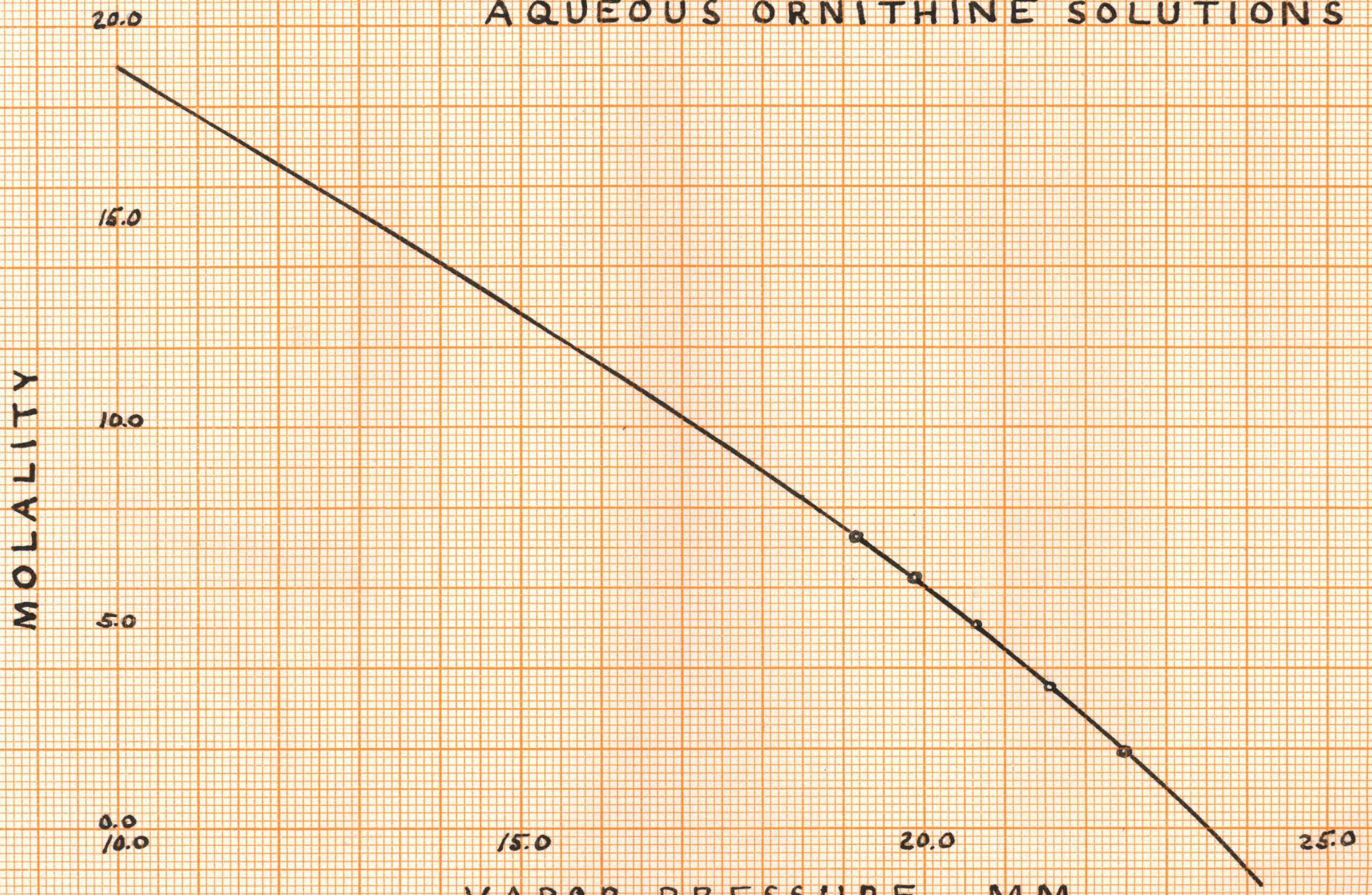
Molality of Ornithine Solution	Isotonic Molality of NaCl Solution	Vapor Pressure
1.990	1.344	22.46 mm.
3.581	2.394	21.56
5.087	3.404	20.62
6.214	4.132	19.89
7.285	4.872	19.11

Activity of Ornithine in Saturated Solution: 8.333

Activity Coefficient : .17

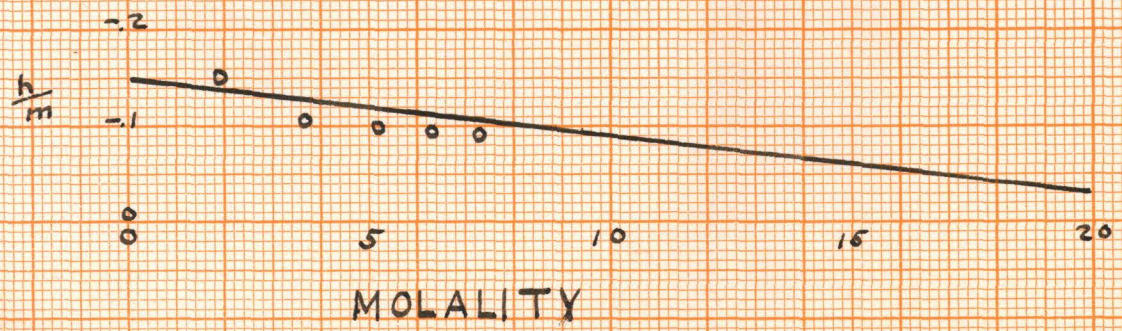
GRAPH VIII

VAPOR PRESSURES OF AQUEOUS ORNITHINE SOLUTIONS



GRAPH IX

ACTIVITY OF ORNITHINE SOLUTIONS



Vapor Pressures of Hydrate Systems

The vapor pressure of citrulline monohydrate in equilibrium with citrulline at 25.0° and of arginine dihydrate in equilibrium with its anhydrous form were measured. This was accomplished by the standard approximation method in which a mixture of hydrate and anhydrous substance is placed in a beaker in an evacuated space with a beaker of sulfuric acid of definite concentration, and gains or losses of weight noted. A series of concentrations of acid are used, which are continually narrowed down to the concentration of acid that remains in equilibrium with the unknown, as shown by the absence of gain or loss in weight over a sufficient period of time, such as forty-eight hours. The vapor pressure of the sulfuric acid solution with which the hydrate-anhydrous pair is found to be in equilibrium is then the vapor pressure of the unknown system.

The desiccators described in the section on activities were employed. The sulfuric acid used was ten times the quantity of hydrate-anhydrous pair. The sulfuric acid was made up from a concentrated acid of known water content (determined from specific gravity) and fluctuations in the acid strength during handling

was noted by finding the concentration of the final aqueous sulfuric acid after each determination from its weight. Desiccators were kept in a 25° incubator during distillation. The method proved sensitive to 0.4 mm. of mercury. Vapor pressures of the sulfuric acid solutions were taken from International Critical Tables (43).

Vapor pressures of the two systems studied are given in Table XIV.

Table XIV

Vapor Pressures

Citrulline - citrulline monohydrate	2.0 ± .4 mm.
Arginine - arginine dihydrate	14.9 ± .4 mm.

Hydrogen-Ion Concentration of Saturated Solutions

The pH's of the saturated solutions were measured on a Beckman pH meter with a glass electrode. The values are given in Table XV.

Table XV

pH's of Saturated Solutions at 25°C.

Solution of	pH
Citrulline	5.90
Arginine	9.45
Ornithine	9.81

Derived Data

In order to obtain the free energy changes of the standard reactions in solution, the procedure described by Borsook and Huffman (6) was followed.

The free energy of formation of the solid compounds was calculated from the heats of combustion and other reference data:

Heats of Formation and Entropies of Elements and Combustion Products

	$H_{298.1}$	$S_{298.1}$	Reference
$H_2O(l)$	-68,313		(44)
$CO_2(g)$	-94,030		(45)
C (graphite)		1.36	(46)
H_2 (g)		31.23	(47)
N_2 (g)		45.78	(48)
O_2 (g)		49.03	(49)

The complete combustion of citrulline at 25° C. involves the reaction:

(a) $C_6H_{13}N_3O_3(s) + 7\ 3/4\ O_2(g) = 6CO_2(g) - 6\frac{1}{2}H_2O(l) - 1\frac{1}{2}\ N_2(g)$

The increased number of mols, N, of gaseous substances from the reaction is $-\frac{1}{4}$

The heat of combustion at constant pressure is

$$\Delta H_p = \Delta U_R - \Delta NRT$$

in which Hp is the heat of combustion at constant pressure and ΔU_R is the heat of combusion at constant volume, corrected to the standard states of the elements.

For citrulline, then,

$$\Delta H_p = 803,320 - (-\tfrac{1}{4})(1.987)(298.1)$$

$$= 803,490 \text{ calories}$$

The heat of formation of citrulline from reaction (a) is

$$803,490 - 6(94,030) - 6\tfrac{1}{2}(68,313)$$

$$= -204,730 \text{ calories}$$

$$S_T = S(\text{Compd},T) - aS(C,T) - bS(H_2,T - cS(O_2,T) - dS(N_2,T)$$

For citrulline, then

$$60 + 6\tfrac{1}{2}H_2 + 1\tfrac{1}{2}N_2 + 1\tfrac{1}{2}O_2 = C_6H_{13}N_3O_3$$

and $S_{298,1} = 60.72 - 6(1.3) - 6\tfrac{1}{2}(31.23) - 1\tfrac{1}{2}(45.78) - 1\tfrac{1}{2}(49.03)$

$$= -290.0 \text{ E.U.}$$

From the equation

$$\Delta F = \Delta H - T\ \Delta S$$

$$\Delta F = -204,730 - 298,1(-290.0)$$

$$= -118,280 \text{ calories}$$

Citrulline forms a monohydrate by the following reaction:

$$C_6H_{13}N_3O_3 + H_2O = C_6H_{13}N_3O_3H_2O$$

For evaluation of energy transfer, this is broken down into the following equations:

(C = Citrulline)

$C + H_2O(g,p) \rightarrow C:H_2O$; $\Delta F = 0$

$H_2O(g,P) \xrightarrow{1} H_2O(g,P_1)$; $\Delta F = RT \ln P_1/P_o$

$C;H_2O \rightarrow C + H_2O$ (sat sol) ; $\Delta F = 0$

H_2O (sat $\overset{aq.sat}{sol}$) $\rightarrow H_2O$ (l) ; $\Delta F = RT \ln P_1/P_s$

H_2O (l) $\rightarrow H_2O(g,P_o)$; $\Delta F = RT \ln P_o/P_1$

By summation

$C \rightarrow C$ (aq.sat.) ; $\Delta F = Rt \ln P1/P_s$

$\Delta F = 1364 \log 2.0/33.11$

$= -1405$ calories

The free energy of formation of the saturated solution is then

$= -118,280 - 1450 = -119,730$ calories.

The free energy change in the transfer from a saturated solution to one in which undissociated citrulline is at 1 Molal activity is then

$\Delta F = -RT \ln 0.8608/1$

$= 89$ calories

The free energy of undissociated citrulline in solution at 1 M activity is then $-119,730 + 90 = -119,640$ calories.

The relative concentration of the Zwitter ion or undissociated form of citrulline is then calculated from the pH of the saturated solution, the dissociation constants, and the following equation:

$$\Sigma \text{Citrulline} = A = (1 + (\overset{+}{H})/K_A + K_W/K_B\ (\overset{+}{H}))$$

where ΣCitrulline is the concentration of citrulline in

all forms. From this it is found that the citrulline exists practically exclusively as zwitter ion.

The above value of -119,640 calories is then the free energy of formation of the neutral molecule at I M activity or

ΔF_o = 119,640 calories.

The corresponding value for arginine was arrived at by using the value of formation of solid given by Huffman and Ellis (50). For the calculation of activity in the case of concentrated solutions obtained by saturation with urea and with ornithine, mol fraction/ mol fraction of 1 M solution was employed instead of molality. The solubility value for urea is from Scatchard, Hamer, and Wood (51).

The value for the free energy of ornithine was arrived at from the study of the arginine-ornithine-urea equilibrium carried out by Hellerman and Perkins (52) and other data . In the work cited, the authors found equilibrium to occur at 98.19% hydrolysis of arginine.

For the reaction

Arginine + water = Ornithine + urea

$$K = \frac{(\text{ornithine})(\text{urea})}{(\text{arginine})}$$

$$K \text{ here} = \frac{(.9819)^2}{(.0181)} = 53.3$$

For this reaction

$$\Delta F = -RT \ln K$$

$$= -1364 \log 53.3$$

$$= -2360 \text{ calories}$$

Then arginine + water = ornithine + urea ; $\Delta F = -2360$ calories Since the free energy change attending a reaction is equal to the sum of the free energies of formation of the products minus the sum of the free energies of formation of the reactants

$$-2360 = -48,440 + X + 58,670 + 56,720$$

from which X, the standard free energy of formation of ornithine = -69,310 calories.

The values for the free energies of formation of ornithine in its various states were then calculated as for citrulline.

The values for ammonia, carbonic acid, and water are taken from the chapter by Borsook and Huffman in Schmidt; The Chemistry of the Amino Acids and Proteins (6).

The assembled values are recorded in Table A.

Table A

The Free Energy of Formation of Undissociated Solute in Standard Solution at 25°C.

Compound	F(sat.sol.)	Activity	F_{1M} undissoc.
d-Arginine	-58,990	.5878	-58,670
Citrulline	-119,730	.8608	-119,640
Urea	-47,200	8.07	-48,440
Ornithine	-68,770	2.50	-69,310
Ammonia (aq.)			-6,300
Carbonic Acid (aq.)			-148,810
Water (l)			-56,720

We can now consider the reactions in the urea cycle of Krebs;

(1) Ornithine + Carbonic Acid + Ammonia

= Citrulline + 2 Water.

ΔF_o at 25° C = -119,640 + 2(-56720) + 69,310 + 148,810 + 6,300

ΔF_o = -8,660 calories

(2) Citrulline + Ammonia = Arginine + Water

ΔF_o at 25°C = -58,670 -56,720 + 119,640 + 6,300

ΔF_o = 10,550 calories

(3) Arginine + Water = Ornithine + Urea

ΔF_o at 25° C. = -2,360 calories calculated above

Reactions (1) and (3) may occur quite readily. The energy for reaction (2) must arise, however, from some other mechanism. Another possible intermediate is the δ N - carboxy ornithine suggested by Schmidt (53).

Other Thermal Data

Preparations and Purifications.

The limiting factor in calorimetry of substances of physiological importance is usually the purity of the compound under investigation. In the case of calorimetric standards such as benzoic acid, and many other simple organic chemicals, high purity is easily attained. The attainment of high purity in many biochemicals is often, however, a difficult chemical problem, requiring much more care and labor than the original synthesis or isolation. In what follows, purifications for combustion values (determined largely by Professor Huffman) will be set out in detail, and in many cases discussed with relation to the combustion values obtained.

d Alanine

Two samples of Hoffman La Roche d-alanine were prepared by recrystallisation from water, and two by recrystallisation from water and ethanol.

Materials from four commercial and one unknown source were utilized in preparing sixteen different combustion samples.

Sample (c) A product obtained from the "Amino Acid Manufactures" was dissolved in boiling water and allowed to crystallize in the cold room. The mother liquor was treated with two volumes of 95% ethanol. The

two fractions thus obtained were combined and were again dissolved in boiling water and allowed to crystallize.

Sample (d) The solute in the mother liquor from (c) was precipitated with two volumes of 95% ethanol.

Sample (e) A portion of (d) was twice recrystallized from water and finally from hot water by the addition of alcohol.

Sample (f) A portion of (e) was dissolved in hot water and allowed to cool. After standing for several days the mother liquor was filtered off and treated with ethanol. The precipitate thus obtained was twice recrystallized from water.

Sample (g) The mother liquors from (c) and (f) were combined and ethanol added. The precipitate thus obtained was twice crystallized from water.

Sample (h) A sample made up of accumulated residues was benzoylated with benzoyl chloride, in chilled aqueous solution, in the presence of an excess of sodium bicarbonate. The benzoylalanine, obtained by pouring the reaction mixture into concentrated hydrochloric acid, was washed with water and boiling carbon tetrachloride and finally twice recrystallized from water. A portion of this benzoylalanine was refluxed with 48% hydrobromic acid. The hydrobromic acid was removed by vacuum distillation on the water-

bath, followed by three additions of water and its subsequent removal by vacuum distillation. The residue was neutralized to litmus with ammonium hydroxide and again evaporated. The residue thus obtained was washed free of halides with absolute ethanol and finally crystallized from water.

Sample (i) Duplicate of (c).

Sample (j) Duplicate of (d).

Sample (k) Some alanine residues were twice recrystallized from water by the addition of methanol.

Sample (l) A sample of Pfansteihl "d-alanine" was decolorized with norite and crystallized three times from water.

Sample (m) A sample of Eastman dl-alanine was decolorized with norite and twice recrystallized from water.

Sample (n) The mother liquor from (m) was diluted to twice its original volume and the alanine precipitated by the addition of two volumes of dioxane. The precipitate was dissolved in cold water and the alanine precipitated by the addition of 2.5 volumes of alcohol followed by 2.5 volumes of dioxane.

Sample (o) Alanine residues were recrystallized four times from seven volumes of a 50% pyridine-water mixture and the crystals finally washed with boiling absolute alcohol.

Sample (p) Alanine residues were twice crystallized from 3 volumes of 5% acetic acid. The crystals were finally washed with boiling absolute ethanol.

Nitrogen determinations on sample (h) gave the theoretical results. Tests with the polarimeter showed this material to be optically inert. In the early preparations it was found that the ash content was high and for this reason it was attempted to obtain an ash-free substance by resorting to crystallizing from various solvents and under various conditions. While the ash content was thus lowered, it was never possible to obtain a sample which was completely ash-free. The average ash content of the preparation which yielded acceptable combustion values was 0.016%, and in the extreme cases 0.04%.

Combustions were made on samples of (a) and (b) which had been dried in phosphorus pentoxide vacuum. These values were low and later determinations showed that this probably was due to incomplete removal of water.

The combustions of samples (c) and (d) also led to low results, which could not be due to moisture content, as samples dried for three days and nine days at 100° gave values which were in excellent agreement among themselves. However, a sample dried for sixty days in phosphorus pentoxide vacuum gave a value nearly 15 calories lower. This difference was just accounted for by the loss of mass

of samples whose history was the same except for additional drying at 100°.

Further purification of samples (c) and (d) led to an increase in their combustion values which were then in excellent agreement with preparations from other sources which yielded acceptable values.

There is, of course, the possibility that the low values of (c) and (d) may be due to a difference in crystal form. For this reason an attempt was made to duplicate these preparations, samples (i) and (j); one successful combustion on (i) was in good agreement with the earlier combustions. Since some of the other preparations were finally crystallized under the same conditions and since they had the same appearance under the microscope it was concluded that these low results were due to impurities.

dl Alanyl Glycine

D. Sample B was crystallised four times from aqueous solution in a vacuum desiccator over NaOH in the presence of ethanol.

E. General residues were recrystallised twice from water by allowing the aqueous solution to stand in a dust-free air draught until crystals separated.

F. Alanyl glycine was synthesized by the following scheme:

$CH_3-CH_2-COOH + Br_2$ (P)

↓

$CH_3-CHBr-COBr + NH_2-CH_2-COOH$ (NaOH)

↓

$CH_3-CHBr-C(=O)-NH-CH_2-COOH + NH_3$

↓

$CH_3-CHNH_2-C(=O)-NH-CH_2-COOH$

With the precautions to be described below it was possible for the first time to obtain an alanyl glycine which had an almost negligible ash content. Freedom from ash is desired for two reasoms:

1. The ash residue as determined by ignition of the platinum crucible after a combustion probably does not represent the true original amount of ash present.

2. Because of the unknown nature of the ash it is not possible to correct for its effect on the heat of combustion.

Alpha brom propionyl bromide was prepared from redistilled propionic acid and bromine in the presence of red phosforus, by gentle refluxing. The product boiling at 150-155° uncorrected was coupled with glycine in the fashion described for brom propionyl chloride by Fischer (54). It was possible to obtain crystals of alpha brom propionyl glycine readily, the necessary precaution probably being the evaporation of all solutions in vacuo below 50°.

The brom propionyl glycine was recrystallised four times from chloroform (7 cc. per gram of compound), until a sample melted at 101-103° uncorrected.

For the amination a flask was used which had stood 11 days with concentrated ammonium hydroxide, and was then washed out. Freshly prepared ammonium hydroxide was used in this flask for the amination.

The alanylglycine obtained was twice dissolved in two parts of hot water and precipitated with several volumes of absolute ethanol.

l-Asparagine Hydrate

Material from four different commercial sources was utilized to prepare eleven combustion samples.

Sample (a) Merck l-asparagine, which had been recrystallized several times for heat capacity measurements, was subjected to three additional crystallizations by dissolving in boiling water and allowing crystals to form as the solution cooled.

Sample (b) A preparation from Hoffmann-LaRoche was used without further purification.

Sample (c) A portion of (b) was once crystallized from water by cooling the hot solution.

Sample (d) A portion of (b) was twice crystallized

from water as in (c).

Sample (e) Material from Pfanstiehl was twice crystallized from water.

Sample (f) Material from Eastman was twice crystallized from water.

Sample (g) A sample from Merck recently purchased, (1936) for this research was twice crystallized from water.

Sample (h) A portion of (a) was converted to the copper salt, which was purified by one crystallization from a larger volume of water. The copper was precipitated with hydrogen sulfide and the regenerated asparagine was further purified by several crystallizations from water.

Sample (i) A portion of (e) was recrystallized by dissolving in hot water and seeding heavily with l-asparagine which had been dehydrated at 100°.

Sample (j) A portion of (e) which had been dehydrated at 100° was rehydrated by allowing to stand several days covered with water at room temperature.

Sample (k) A part of (a) was twice more crystallized from water.

The nitrogen content of (a) was the theoretical. Measurements of the rotation of samples (a) and (b) in HCl solutions with the ratio (HCl/asparagine hydrate) equal to 1.3 gave values for $[\alpha]^{20}D$ of 30.3 and 27.9,

respectively. The ash content of all the samples with the exception of (a), (h) and (k) was undetectable. In sample (h), the worst case, it was less than 0.01%.

Numerous combustions on sample (a) dehydrated at 100° consistently gave results about five calories higher than the accepted value. For this reason preparations (h) and (k) were made. The result of one combustion on (k) agreed with those on (a) while the result of one combustion on (h) was about midway between those on (a) and the accepted value. Further attempts at purification were impossible because of lack of this material. When a new sample (g) was purchased from Merck it was found to yield values which agreed with the accepted result.

Benzanilide

$$C_6H_5COCl + NH_2C_6H_5(NaOH)$$
$$\downarrow$$
$$C_6H_5\text{-}CONH\text{-}C_6H_5$$

Fifty ml technical aniline were emulsified with 500 ml. 2N NaOH. While the temperature was maintained below 10°, 58 ml. benzoyl chloride were added with continued stirring during 1 hour. The mixture was then acidified to Congo Red with HCl, and the benzanilide filtered off, and washed with water.

This product was twice crystallised from approximately

13 cc. of 95% ethanol per gram of product.

A sample melted at 161-2°.

Creatine (Anhydrous)

Two commercial products were utilized to prepare three different combustion samples.

Sample (a) Eastman creatine was twice crystallized from water by dissolving in boiling water and cooling the solution.

Sample (b) Creatine from Hoffmann-LaRoche was twice crystallized from water as above.

Sample (d) A portion of sample (a) was dissolved in boiling water and crystallization started by seeding with anhydrous creatine which had been dehydrated at 100°.

The nitrogen content was theoretical and the ash content negligible.

Creatinine

Materials from two commercial sources were utilized to prepare four combustion samples.

Sample (b) A portion of the starting material in (a) was converted to the hydrochloride and neutralized with ammonia according to Edgar and Hinegardner's methods (a) and (b). It was finally washed with ammonia and ethyl alcohol.

Sample (c) A sample from Eastman was dissolved in

water and precipitated with acetone as in (a).

Sample (d) A sample of Pfanstiehl creatinine especially purchased for this research (1936) was dissolved in boiling water and immediately placed in an ice-bath to crystallize. The crystals were slightly greenish. This material was again dissolved in hot water and treated with norite. The crystals from the above were again dissolved in hot water and allowed to cool and crystallize.

Nitrogen determinations on (a) gave theoretical values. Sample (c) was found to have a high ash content, 0.02-0.03%, while that of sample (a) and (d) was negligible.

Alpha Dextrose

The method of Hudson and Dale was used to purify and convert glucose, from two different sources, into α-dextrose. A preparation obtained using Pfanstiehl c.p. glucose as the starting material was washed with boiling absolute alcohol. This treatment apparently caused mutarotation, as this material had an initial specific rotation of 62°, sample (a). A portion of this sample was again converted to the α-form and was washed twice by shaking with about 5 volumes of 80% etnanol followed by rapid filtration to give sample (b). Sample (c) was prepared from Kahlbaum dextrose in the same

manner as sample (b). In this case it was necessary to use norite to decolorize.

Sample (d) was made from Pfanstiehl c.p. dextrose by dissolving in one-sixth its weight of hot water. This concentrated solution was then allowed to cool slowly to 75° with continueus stirring. Under these conditions the stable crystalline modification is anhydrous α-dextrose. The mass thus obtained was transferred to a suction filter in an air-bath at 80°. No visible filtration took place but in about one hour the mass was superficially dry.

Bureau of Standards dextrose (standard sample no. 41) was used without further purification, sample (e).

The initial specific optical rotation was determined on samples (b), (c), (d) and (e) giving for $[\alpha]^{25}D$ 113, 113, 109 and 110°, respectively. The ash determination on each of these samples showed that this factor was entirely negligible.

Beta Dextrose

The procedure of Hudson and Dale (55) was followed with purified Pfanstiehl dextrose. An immediate precipitate of beta dextrose was however not observed and on crystallisation 800 cc. absolute ethanol instead of 500 cc., were used. After two such recrystallisations, $[\alpha]_D = -19.5°$. Sample (a).

Sample (b) A purified batch of combined Pfanstiehl and Kahlbaum glucose was converted to the beta form by the Hudson and Dale procedure. Instead of being recrystallsed, twenty grams of yet moist material was swirled for two minutes with 50 cc. of 80 percent ethanol, filtered rapidly, and the product swirled for one minute with 40 cc. of ethanol. The product from this was similarly treated with 15 cc. of alcohol for 1/2 minute. The final product was immediately placed in vacuo over P_2O_5.

$[\alpha]_D = -25^\circ$.

Sample (c) Residues of Kahlbaum dextrose were converted to the beta form by the Hudson and Dale procedure. Two crystallisations were effected.

$[\alpha]_D = -19^\circ$.

Sample (d) One hundred grams of Baker's c.p. dextrose was dissolved in 200 cc. of redistilled dried pyridine previously brought to boiling. The solution was rapidly filtered through a Buchner funnel. After two days' standing, the crystals were filtered off, washed well with ether, dried in the air a few minutes, and then set over sulfuric acid in vacuo overnight. The material was now set in the oven at 105°. It was next pulverized and heated for a few hours more.

$[\alpha]_D^{23} = -21.5^\circ$.

Sample (e) Sixty-five grams of sample (d) was dissolved in 65 cc. of ice water, filtered rapidly, and treated with 325 cc. of absolute ethanol with seeding and mechanical stirring for one hour, and then dried in vacuo over P_2O_5. The 36 gm. obtained was similarly treated with 35 cc. of ice water and 250 cc. of ethanol. The 25 grams next obtained was similarly treated with 25 cc. of ice water and 150 cc. of ethanol.

$[\alpha]_D^{26} = -19.5°$.

Sample (f) The process of sample (a) was repeated, with purified Pfanstiehl dextrose, except that recrystallisations were rapid, aided by mechanical stirring, and only 5 volumes of ethanol per volume of water, were employed.

$[\alpha]_D^{27} = -20.0°$.

Sample (g) The process of sample (a) was closely followed.

$[\alpha]_D^{27} = -20.0°$.

Allowing a part of (g) to stand in the mother liquor 5 days did not improve the poor microcrystalline appearance.

Alpha Dextrose Hydrate

Pfanstiehl dextrose was purified and converted to α-dextrose in the manner previously described. It was covered with 80% ethanol, allowed to stand for nine days,

the ethanol filtered off and the crystalline material dried in the air of the room. The specific rotation of this material, based on the weight of hydrate, was 101°. The ash content was negligible.

Fumaric Acid

Samples (a) and (b) were prepared from Eastman practical fumaric acid by 2 and 3 crystallizations from water in the usual manner. Sample (c) was prepared originally by Professor J.B. Conant of Harvard University and was used in the heat capacity investigation of Parks and Huffman (56). For this investigation it was subjected to two additional crystallisations from water. Titration of sample (c) with sodium hydroxide gave the theoretical value.

Glutathione

Samples of glutathione were crystallised and their ash content lowered by the following procedure: Crude glutathione was dissolved in ten parts of water at room temperature, filtered, and set in a vacuum desiccator over flake NaOH and a dish of ethanol. The water distilled to the NaOH and the ethanol gradually distilled to the dish containing glutathione. Microcrystalline prismatic needles were obtained.

Glycyl Glycine

Glycyl glycine, the following two compounds,

benzanilide, alanyl glycine, and leucyl glycine were all prepared for evaluation of the free energy of formation of the peptide bond.

Sample (a) Twenty grams of A.P. (Amino Acid Manufacture) glycyl glycine was twice recrystallised from two parts of water.

Sample (b) By evaporation of the mother liquor of sample (a) there was obtained a residue of glycyl glycine which was dissolved in 50 cc. of boiling water and treated with 150 cc. of absolute ethanol.

Hippuric Acid

Sample (a) Forty grams of hippuric acid (Amino Acid Manufactures A.P., used in Cp determinations) was recrystallised from 1300 cc. of water.

Sample (c) Eastman hippuric acid was crystallised twice from 35 parts of boiling water.

Sample (e) Hippuric acid(Amino Acid Manufactures) was recrystallised 5 times from 35 parts water.

Sample (f) The first crystallisation mother liquors of sample (e) were evaporated to dryness and the residue recrystallised from 16 cc. of boiling absolute ethanol per gram of material.

Sample (g) Pfanstiehl hippuric acid was recrystallised

from 31 parts of water.

Sample (h) This was the same as (f) except the last 3 recrystallisation liquors of (e) were employed.

Sample (i) Sample (h) was recrystallised from 35 parts of water.

Hippuryl Glycine

Sample (a) Hippuryl glycine (Amino Acid Manufactures, A.P.) was recrystallised from 19 parts of water.

Sample (b) A portion of sample (a) was dissolved in 19 parts of boiling water, treated with a mutual solution of 2 parts ethanol and 2 parts pentanol, cooled, the crystals filtered off, and washed with absolute ethanol.

Sample (c) Sample (b) was recrystallsed from 19 parts of water.

Sample (d) Some Amino Acid Manufactures, A.P. hippuryl glycine was recrystallised from 17½ parts of water, treated with 17½ parts of boiling H_2O for 1 hour, cooled, filtered, and again recrystallised from H_2O. This procedure was intended to reveal any hydrolysis during recrystallisation.

Sample (e) This was a duplicate of sample (b) with a second purchased quantity of hippuryl glycine.

Sample (f) A portion of the new batch was recrystallised from 20 parts of water.

l-Hydroxyproline

The crude, brown hydroxyproline obtained by Bergmann's procedure (57) was obviously not pure enough for determination of thermal constants. By following the directions which outlined the removal of l-proline from arginine flavianate mother liquors as the rhodanilate, and the subsequent precipitation of hydroxyproline as the reineckate, it was possible to obtain approximately the recorded yield of hydroxyproline. Attempts to decolorise with norit were unsuccessful, but a beautiful product was obtained by forming the picrate, recrystallising,and recovering the hydroxyproline after decomposition with acid.

l-Leucine

Preparation from Hoffmann-LaRoche, Carlsberg Laboratory and from the resolution of dl-leucine were the starting materials for eleven combustion samples.

Samples (a), (b), (c), (d), (e) Numerous attempts were made by recrystallisation, etc., to obtain samples of Hoffmann-LaRoche l-leucine free from sulfur but without succeeding.

Sample (f) Hoffmann-LaRoche l-leucine was formylated by the Steiger (58) method. The formyl-l-leucine thus obtained was crystallised six times from water. It was then

hydrolysed with hydrochloric acid and neutralised with lithium hydroxide according to the method of Fischer and Warburg (28). The crystals thus obtained were washed with ethanol.

(g) The l-leucine obtained in the resolution of dl-leucine, described under d-leucine, was recrystallised similarly from water.

Sample (h) This sample was obtained by evaporating the mother liquor from (g) in vacuo until crytals appeared.

Sample (i) A portion of (f) was once recrystallised from water.

Sample (j) A collection of l-leucine residues was formylated by the method of Fischer and Warburg (28) for synthetic leucine. The formyl-l-leucine thus obtained was recrystallised from water six times. It was hydrolysed with hydrobromic acid and neutralized with ammonium hydroxide in the manner already described. The material from the above treatment was recrystallised once from water.

The nitrogen analyses on the above samples were the theoretical. Sulfur analyses were completely negative. The rotation, detemmined on a solution containing 20% hydrochloric acid and with a ratio of hydrochloric acid/ leucine of 25, for sample (h) was $[\alpha]^{23}_{D}$ -16.5° and on sample (i) $[\alpha]^{23}_{D}$ -15.3°. The ash content of the final preparations was less than 0.01%. Carbon appeared in the

crucible occasionally but was negligible in amount.

d-Leucine

The residue from the dl-leucine preparations were utilized as the starting material. These were formylated by the method of Steiger (58) in a formylation apparatus kindly loaned to us by Professor M.S. Dunn. The resolution was made according to the method of Fischer and Warburg (28) with the following modifications in the hydrolyses. The formyl-d-leucine was refluxed for one hour with fifteen times its weight of 10% hydrobromic acid. The solution was evaporated in vacuo with the subsequent addition of water three times, neutralized to litmus with concentrated ammonium hydroxide, filtered, and the leucine washed with absolute ethanol until halide free.

Sample (a) The d-leucine obatined by the above method was recrystallized once from water.

Sample (b) The mother liquor from (a) was concentrated by pumping off a part of the water and a second crop of crystals obtained.

The nitrogen was found to be the theoretical. The rotation, determined with a solution containing 20% hydrochloric acid and with a ratio of hydrochloric acid/ leucine of 25 gave $[\alpha]^{24}_{D}$ -15.7°. The ash content was negligible and in only one case was there any evidence of carbon.

Maleic Acid

Sample (a) was prepared originally by Professor J. B. Conant from maleic anhydride and was used by Parks and Huffman for heat capacity measurements. For this investigation it was twice recrystallized from 50% aqueous solution.

Sample (b) was obtained by four crystallisations of Pfanstiehl C.P. maleic acid. This sample gave low combustion values.

Samples (c) and (d) were prepared from Eastman maleic anhydride. Two portions of this material were each subjected to three fractional distillations giving final products boiling at 200° and having boiling ranges of 0.3 and 0.1°, respectively. The first distillate was dissolved in ten parts of water and evaporated to dryness over sodium hydroxide (c). The second distillate was dissolved in three parts of water and evaporated to dryness over phosphorus pentoxide (d). The ash content was completely negligible.

Phenylthiourea

Sample (a) Eastman phenylthiourea was twice recrystallised from twenty parts of water.

Sample (b) A batch of material purified as for sample (a) was dissolved in 40 parts of 95 percent

ethanol, and allowed to evaporate to dryness.

l-Proline

l-Proline was prepared by the method of Bergmann (57) and washed well with absolute ethanol.

$[\alpha]^{18}_D = -84.2^\circ$.

Sample (a) A portion was dissolved in 10 parts of boiling 95% ethanol, solution decanted from a small amount of insoluble matter, and treated with 5 volumes of ether. The resultant crystals were filtered off, and dried in vacuo over P_2O_5.

Sample (b) 4.6 grams of l-proline were dissolved in 150 cc. of boiling absolute ethanol, and added to a solution of 8.1 grams of Baker's C.P. copper acetate in 300 cc. of boiling absolute ethanol, and the solution was filtered. The liquid was evaporated in vacuo almost to dryness, dissolved in boiling absolute ethanol and precipitated with ether. The precipitate was filtered off and again recrystallised in the same way. The resultant copper salt was suspended in water, which was saturated with H_2S. The residue obtained by evaporating the filtrate from CuS and noriting was recrystallised from 95 percent alcohol and ether, and then from ten parts of n-propyl alcohol.

Sample (c) The combined mother liquors of sample (a)

were evaporated in vacuo and the proline residue was recrystallised from 150 cc. of n-propyl alcohol, then from 120 cc., then from 110 cc., and finally from 100 cc., with noriting.

Unsuccessful attempts to remove the odor included heating overnight at 100°, dissolving in cold alcohol and precipitating, placing in vacuo for many hours, and sublimation. Recrystallisation from n-propyl alcohol effected the difficult removal of colored inorganic salts.

dl-Pyroglutamic Acid

d-Glutamic acid was heated for one hour in a beaker on a sand-bath with the temperature of the melt at 170-195°. It was stirred while cooling and then an equal volume of ethanol was stirred in. The crystals which resulted were recrystallised from water, and from water and ethanol with noriting.

Succinic Acid

Succinic acid was recrystallised four times from 2.3 times its weight of water at 70°.

Taurine

Taurine was synthesized by the following steps:

$NH_2-CH_2-CH_2-OH + HBr$

$\downarrow$

$NH_2-CH_2-CH_2-Br$

$\downarrow \qquad + Na_2SO_3$

$NH_2-CH_2-CH_2-SO_3H$

The directions were those given by Cortese (59) and the product was recrystallised twice in the manner described in his paper, for entropy determination. This material was halide-free.

Sample (a) For combustion, forty grams of material was recrystallised from 160 and 120 cc. of water.

Sample (b) The mother liquors of (a) were combined and treated with 1400 cc. of ethanol. The crystals which separated were dissolved in 60 cc. of hot water and 300 cc. of ethanol were added.

Sample (c) Ten grams of Hoffmann-LaRoche taurine was recrystallised from 40 cc. and 28 cc. of boiling redistilled water.

l-Tyrosine

One commercial material and a student preparation from silk were used in making five different combustion samples.

Sample (a) A sample from Hoffmann-LaRoche was used without additional purification.

Sample (b) A portion of (a) was once recrystallised from hot water.

Sample (c) The student preparation was once recrystallised from water.

Sample (d) A portion of (a) was twice recrystallised

from 275 parts of water.

Sample (e) The student preparation was treated with norite and recrystallised three times from 250 parts of water.

The nitrogen content was found to be the theoretical. The rotation was determined on a solution containing 0.863 gm. of tyrosine in 25 cc. of 4% hydrochloric acid and gave $[\alpha]^{24}_{D}$ -10.8. The ash content of all of the preparations except (a) was entirely negligible. With one exception there was no evidence of the production of carbon and in this case the correction was less than 0.01%.

Entropies

Entropies were determined as described in Section I for citrulline and ornithine. They were determined for the following substances:

Creatine Hydrate

Glycyl Glycine

Hippuric Acid

Hippuryl Glycine

l-Proline

Taurine

The values of the heat capacities and entropies are presented in the Tables immediately following.

Table XVI

Specific Heats of Creatine Hydrate

Temp °K	Calories/gram/degree
87.3	.1326
92.0	.1380
97.2	.1435
103.2	.1507
109.7	.1576
116.5	.1649
136.3	.1852
155.5	.2051
172.8	.2222
190.1	.2402
210.0	.2600
226.3	.2749
242.9	.2909
258.8	.3052
273.6	.3189
279.9	.3259
285.6	.3306
292.2	.3366
298.4	.3423

GRAPH X

SPECIFIC HEATS OF CREATINE HYDRATE

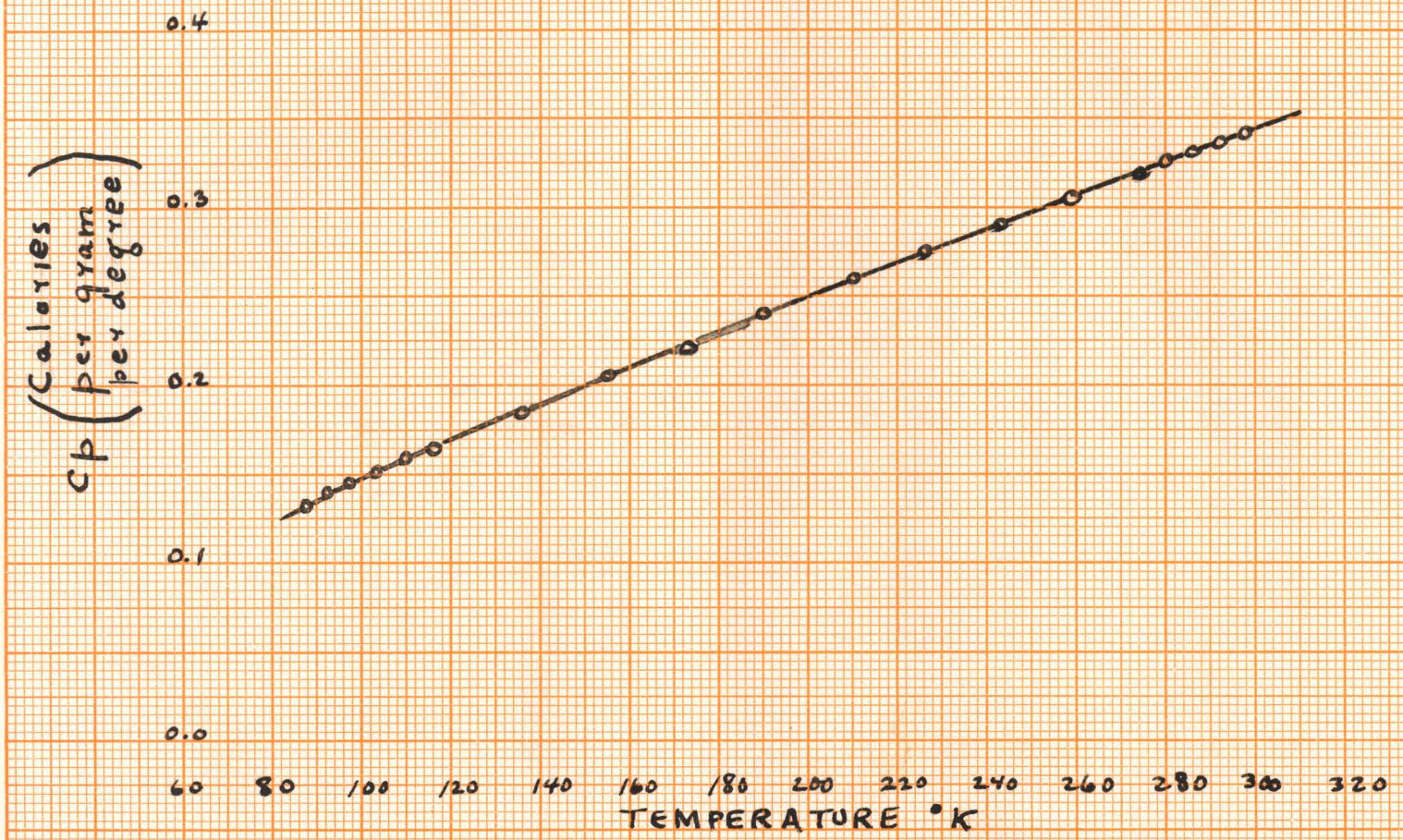


Table XVII

Specific Heats of Glycyl Glycine

Temp ^{o}K	Calories/gram/degree
86.7	.1239
91.8	.1292
97.9	.1355
103.6	.1417
110.4	.1486
117.2	.1561
136.0	.1734
155.4	.1894
172.8	.2040
190.5	.2168
209.5	.2308
223.5	.2403
244.1	.2555
257.6	.2650
276.2	.2784
280.3	.2818
287.2	.2877
293.9	.2927

GRAPH XI

SPECIFIC HEATS OF GLYCYL GLYCINE

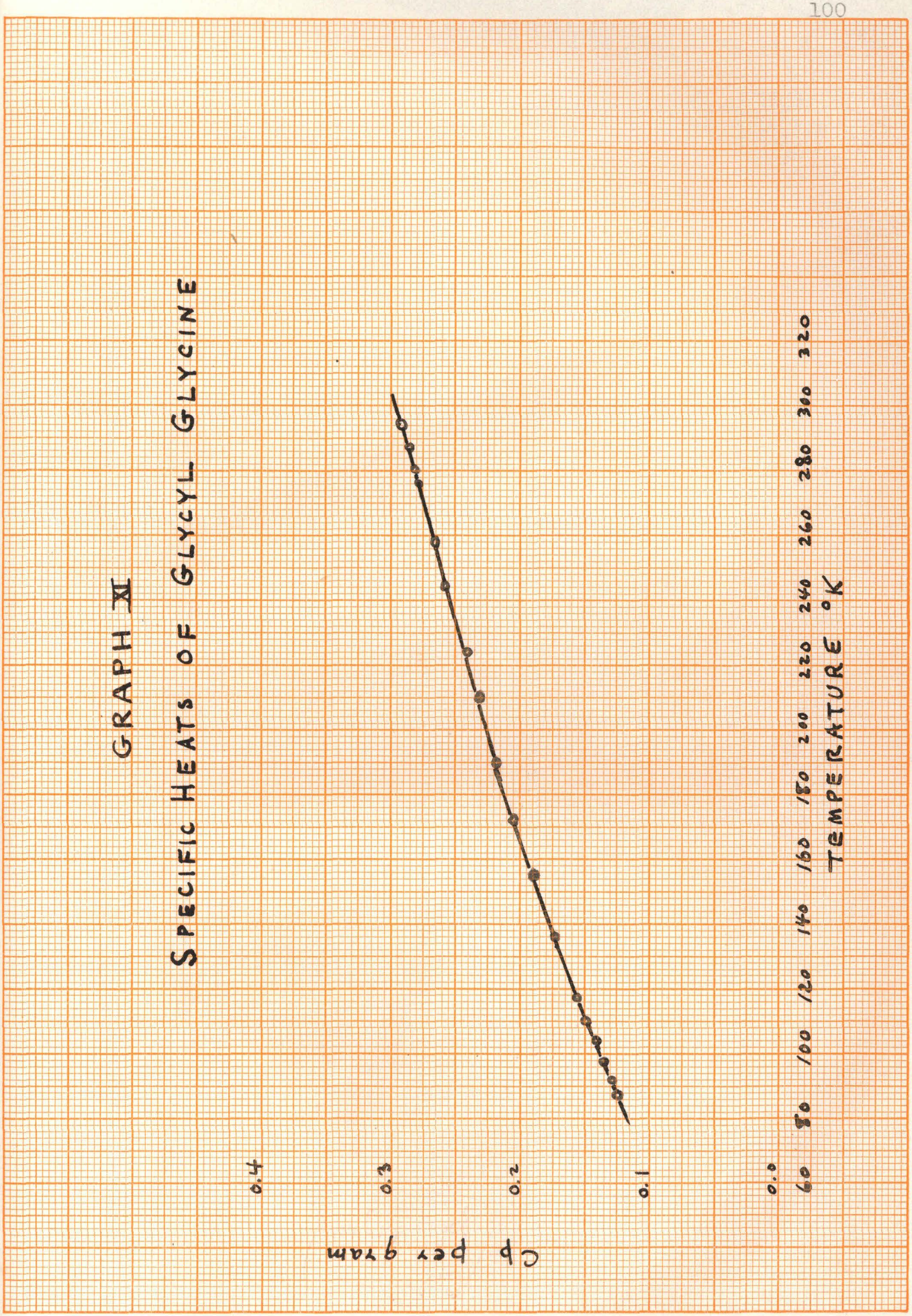


Table XVIII

Specific Heats of Hippuric Acid

Temp °K	Calories/gram/degree
84.8	.1075
89.6	.1111
95.6	.1160
103.1	.1228
111.0	.1292
120.1	.1369
129.6	.1440
145.1	.1562
160.0	.1680
174.7	.1798
190.2	.1936
205.7	.2058
220.2	.2186
240.4	.2348
260.2	.2519
275.8	.2658
281.1	.2707
287.7	.2767
293.3	.2814
298.4	.2859

GRAPH XII

SPECIFIC HEATS OF HIPPURIC ACID

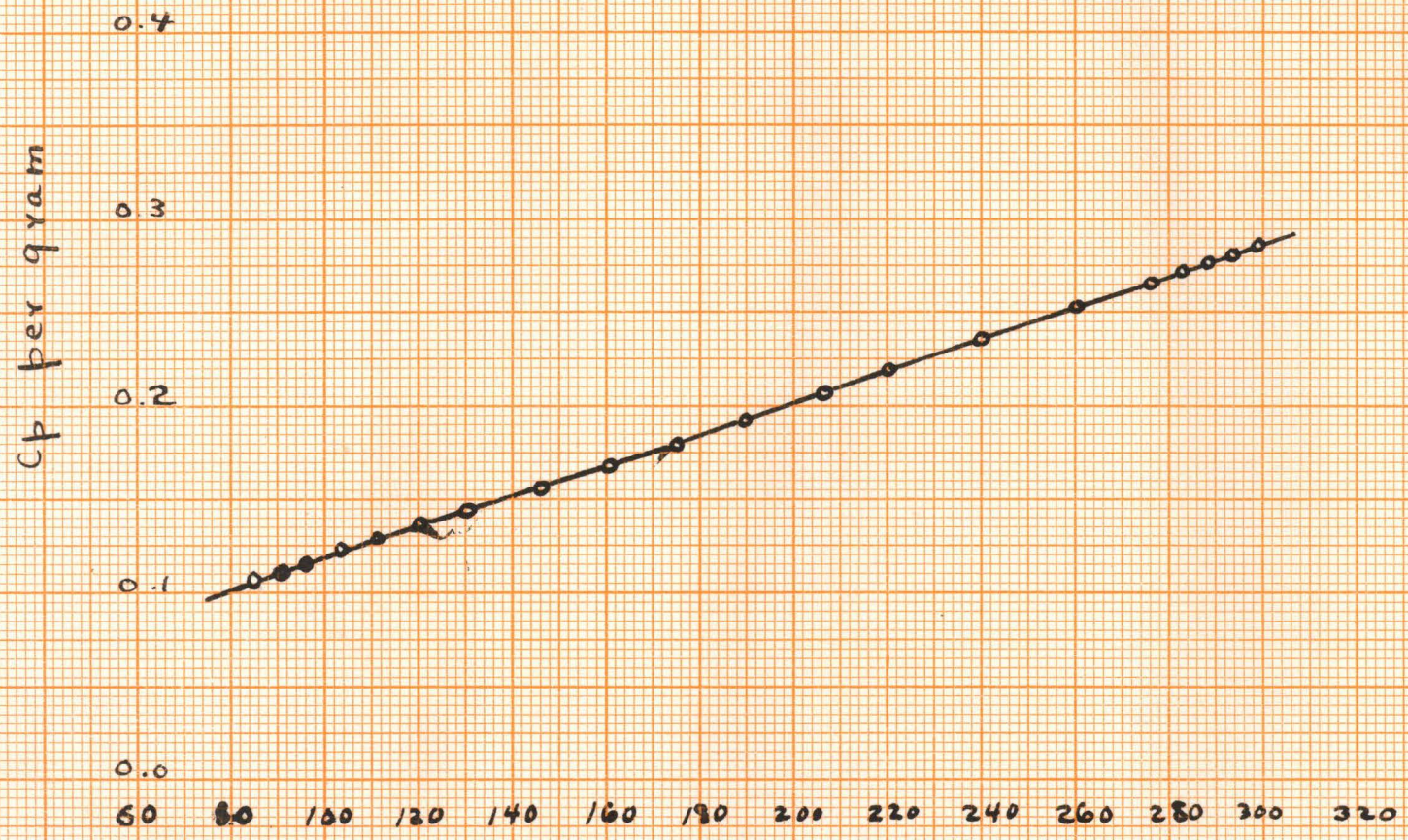


Table XIX

Specific Heats of Hippuryl Glycine

Temp °K	Callories/gram/degree
84.7	.1066
87.7	.1088
93.6	.1136
94.6	.1152
100.3	.1201
102.8	.1223
112.2	.1304
122.7	.1414
133.1	.1513
134.1	.1520
147.2	.1622
156.4	.1682
160.7	.1707
180.7	.1854
185.9	.1896
198.0	.1991
205.9	.2052
216.7	.2140
228.7	.2236
241.5	.2337
253.7	.2438
262.2	.2508
265.7	.2534
277.3	.2638
281.7	.2673
287.2	.2716
296.7	.2808

GRAPH XIII

SPECIFIC HEATS OF HIPPURYL GLYCINE

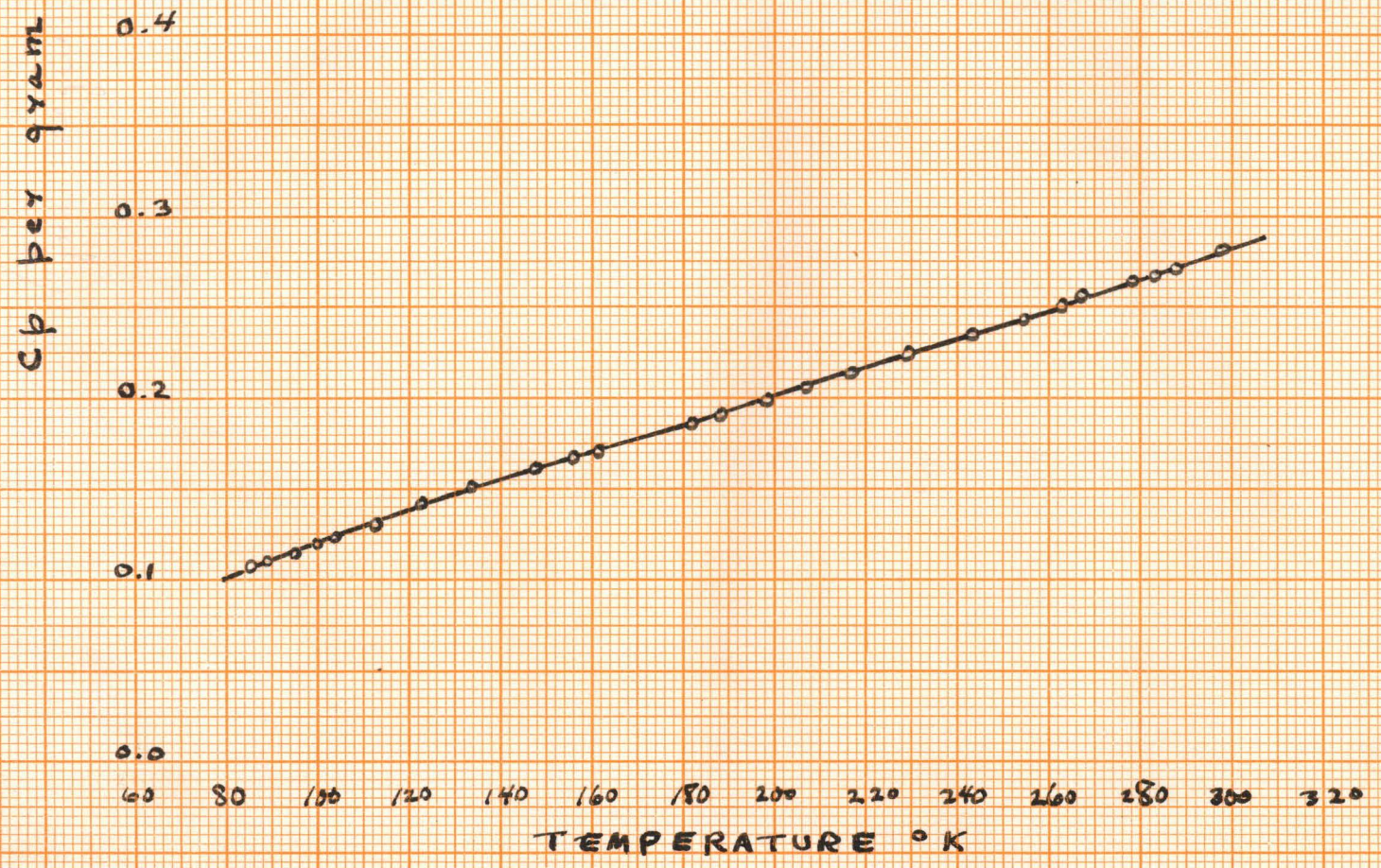


Table XX

Specific Heats of l-Proline

Temp °K	Callories/gram/degree
87.7	.1223
94.8	.1286
101.3	.1348
108.3	.1411
116.5	.1478
125.1	.1555
154.2	.1811
171.8	.1969
189.1	.2119
204.0	.2257
219.2	.2390
237.3	.2549
247.0	.2630
257.5	.2724
276.8	.2892
283.2	.2947
291.8	.3027
300.4	.3096

GRAPH XIV

SPECIFIC HEATS OF L-PROLINE

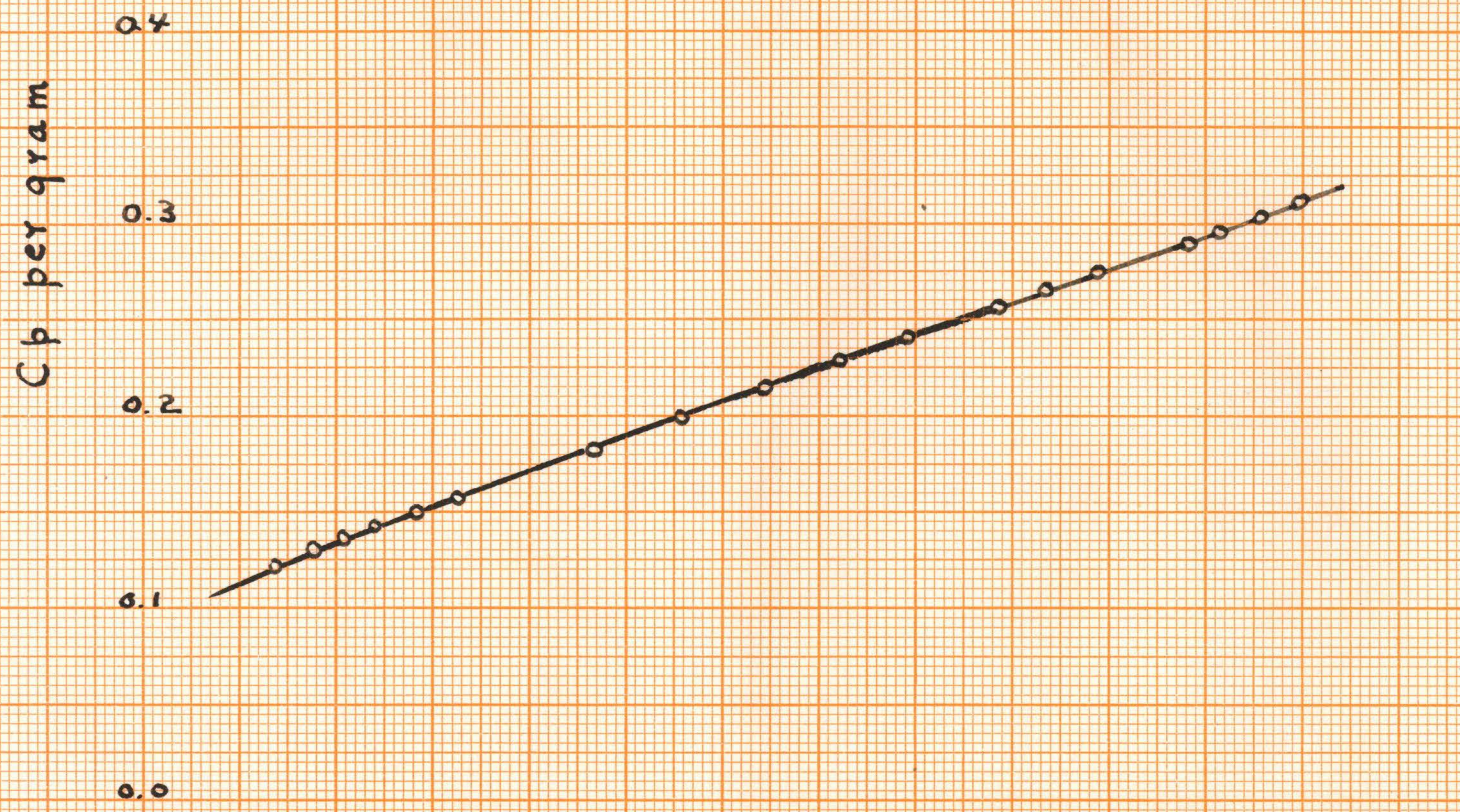


Table XXI

Specific Heats of Taurine

Temp °K	Callories/gram/degree
87.4	.1031
92.8	.1080
97.9	.1122
112.6	.1263
123.9	.1361
140.7	.1503
159.7	.1649
178.1	.1790
193.4	.1909
208.0	.2018
221.9	.2116
236.4	.2215
249.9	.2304
263.7	.2402
276.3	.2484
281.8	.2523
293.0	.2605
300.3	.2659

GRAPH XV

SPECIFIC HEATS OF TAURINE

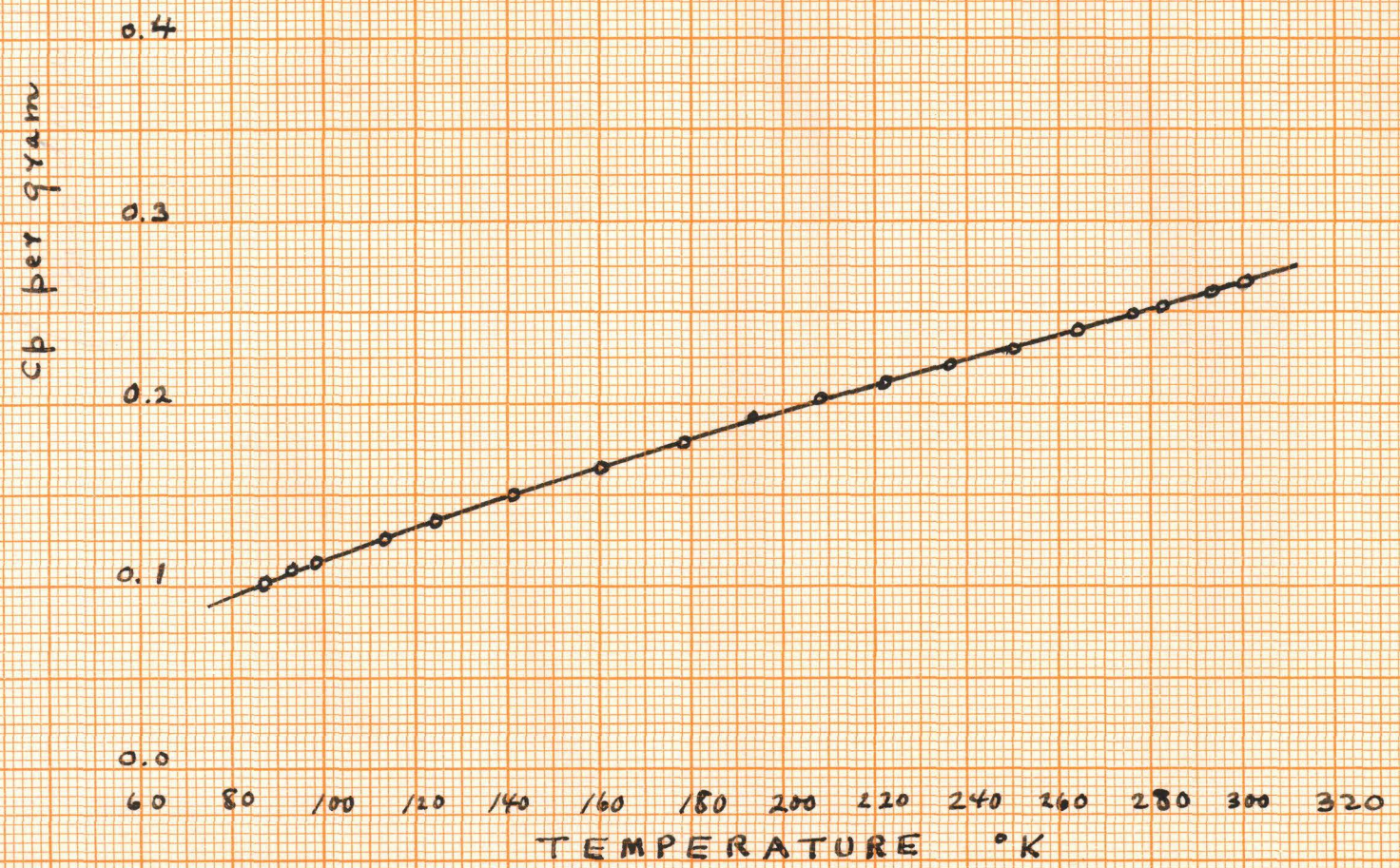


Table XXII

Entropies of the Compounds

(E.U. per mol)

Substance	S_{90°	$S_{90^\circ-298.1^\circ}$	$S_{298.1^\circ}$
Creatine Hydrate	16.39	39.57	55.96
Glycyl Glycine	13.57	31.73	45.30
Hippuric Acid	18.51	38.68	57.19
Hippuryl Glycine	24.14	50.96	75.10
l-Proline	13.40	27.37	40.77
Taurine	10.52	25.91	36.43

Heats of Combustion

Heats of combustion of fumaric and maleic acids were determined as described for citrulline and ornithine in Section I. The values are presented in Tables XXIII and XXIV.

Table XXIII

Heats of Combustion of Fumaric Acid

(15^{o} calories per gram at $25^{o}C$)

Sample	$-U_B$
(a)	2754.4
(a)	2754.7
(b)	2754.3
(c)	2754.5
(c)	2754.8
(c)	2754.8
(c)	2754.2
Mean	2754.8

Table XXIV

Heats of Combustion of Maleic Acid

(15° calories per gram at $25^{\circ}C$)

Sample	$-\Delta U_B$
(a)	2802.3
(c)	2801.3
(c)	2801.5
(c)	2801.6
(d)	2802.3
(d)	2802.0
Mean	2801.8

III A STUDY OF SPERM AGGLUTINATION

Introduction

While the study of the urea cycle provided knowledge of the detailed metabolism of protein, the physics and chemistry of sperm agglutination presented an opportunity for study of protein in a grosser aspect. Most of the biological experiments carried out in this connection were done jointly with Professor Albert Tyler.

The purpose of the undertaking described here was the chemical characterisation of the egg-borne substance which has the property of agglutinating sperm. The substance has been called "fertilizin" by F. R. Lillie (60). It is referred to as agglutinin in the following pages.

The sperm agglutination reaction, first described in detail by Lillie (61), is a characteristic clumping of sperm resulting when the sperm are placed in sea water in which eggs of the test animals have stood. This clumping is non-toxic, species-specific, and spontaneously reversible. It is, according to Lillie, to be distinguished from the loose clustering caused by CO_2, and from "heteroagglutination", a toxic, non-reversible agglutination in which sperm of another species are caused to clump tightly. Egg water also increases the activity of the spermatozoa.

It is clear that this type of phenomenon has many characteristics in common with bacterial and serological agglutination. Fertilizin has in fact been likened to a "complement" between egg and sperm receptors (62).

Biological Properties

The local marine animals which have been found to exhibit an agglutination reaction are:

Giant Key-hole Limpet (Megathura crenulata)

Sea-worm (Urechis caupo)

Sea-urchin (Strogylocentrotus purpuratus) (Previously described (63)).

Sand-dollar (Dendraster excentricus)

The intensity of the reaction is greatest in the order listed. Since Urechis reaction does not give spherical clusters, there is some question as to its being typical. Very dilute suspensions of sperm (barely milky) will give a reaction in the case of the limpet. It was found, however, that with sea-urchins, relatively undiluted sperm suspensions were necessary for a perceptible agglutination. With higher concentrations of reagents the reversal of reaction took longer. In the most favorable cases, agglutination persisted for many minutes. The Urechis reaction could be made to last for at least 15 minutes. The longest reactions with sand-dollar sperm and egg-water were for a fraction of a minute.

A number of attempts to obtain agglutination of sperm of the little limpet (acmea) with egg-water of the Key-hole limpet were unsuccessful. Efforts to obtain a reaction within the acmea group, by intertesting the sex-products

of about a dozen individuals, at different seasons, were also fruitless.

The Key-hole Limpet reaction itself does not spontaneously reverse, in contrast to the agglutination reaction of the other marine forms. The clumps initially formed gradually approach each other and rather suddenly fuse. That the lack of reversibility is not a concentration effect was shown in the following way: Egg-water solutions sufficiently dilute to give the least perceptible reaction were added to dilute sperm suspensions. The egg-water employed was diluted 10 times with sea-water and gave the first perceptible agglutination in 5 minutes. After 18 hours no reversal had occurred. Another, strongly agglutinated sperm sample, when squirted with a pipet, broke up, but reformed again in many seconds, although not as strongly.

In order to discover if the blood contained agglutinin, 5 drops of blood of a female sea-urchin were added to one drop of milky sperm. Five drops of the blood of the male were also tested. To other samples of the same sperm suspension was added blood of another male and of another female. In no case was agglutination observed. Addition of egg-water to sperm from the animals of this group in all cases produced satisfactory agglutination. This is in agreement with previous work (64).

To test cross-agglutination in the series of animals used, sperm suspensions and egg waters of the limpet, sea-urchin, Urechis, and sand-dollar were made up. The following combinations were tried:

Egg-water	Sperm
Sand-dollar	Sea-urchin
Sand-dollar	Urechis
Sea-urchin	Sand-dollar
Sea-urchin	Urechis
Sea-urchin	Limpet
Limpet	Sea-urchin

In all cases the reaction was negative.

Bio-Assay

Lillie's method for assaying the agglutinating activity of egg waters consists of testing a series of dilutions against samples of one sperm suspension (64). The dilution to which a solution must be brought in order to give a clumping which lasts for but five to six seconds is then a measure of the strength of the solution. Although this has been successfully used on Arbacia, the weaker reaction obtained with the S. purpuratus made it practically difficult to determine when a short-lasting reaction had occurred.

Since a bio-assay was essential as a preliminary step to isolation of agglutinin, other means of quantitative assay of the reaction were sought. Attempts to centrifuge clumps on the assumption that they had a density greater than that of the non-clumped milieu, failed to give perceptible stratification.

The next method tried was that of fixing an agglutinated suspension with Bouin's fluid and counting clumps above a minimum magnitude on an hemocytometer slide.

These experiments were begun with the sea-urchin reaction. It was soon found that a high proportion of Bouin's fluid itself caused clustering, and this factor had to be adequately controlled. Another factor which

required regulation was the strength of the sperm suspensions, stronger suspensions giving higher density of larger clumps. In a comparative series of experiments, this could be adequately controlled by using the same suspension throughout.

At this stage sea-urchins became unavailable, so the technic was transferred to Urechis. To illustrate the effect of egg water dilution, the following experiments were set up:

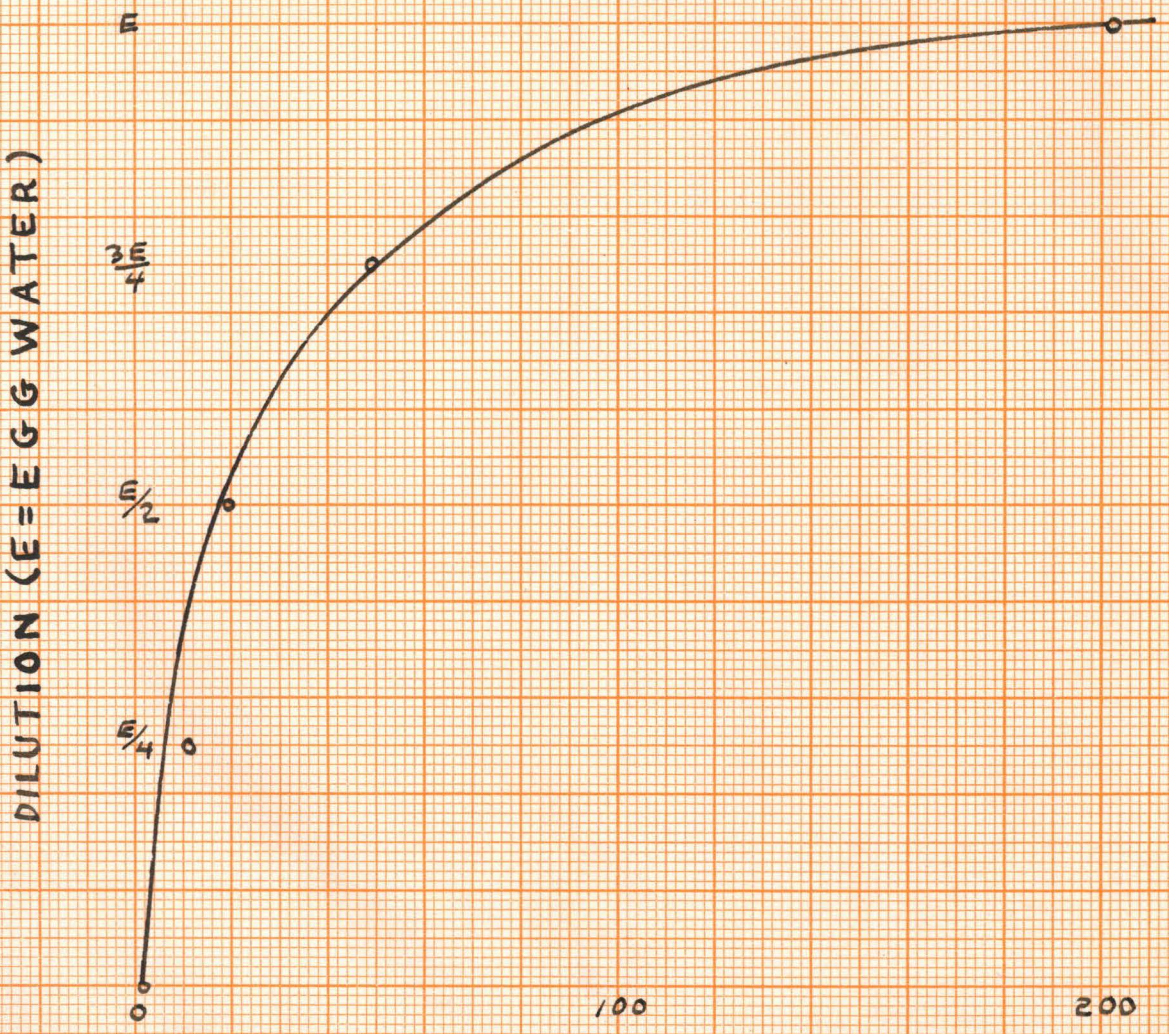
0.25 cc. of sperm and 0.25 cc. of egg water were swirled in a small dish, 0.05 cc. of Bouin's fluid were immediately added, the dish again swirled, and a drop of liquid transferred to the hemocytometer slide. All clumps larger than ½ of the smallest square on the slide were counted.

E=Egg water

Dilution	No. of Clumps
E	202
$\frac{3E}{4}$	49
$\frac{E}{2}$	19
$\frac{E}{4}$	11
Sea Water	2

GRAPH XVI

URECHIS REACTION



Since limpets offered a more convenient bio-assay and probably a richer source of agglutinin and since they became sexually ripe at this stage of the study, another bio-assay was developed.

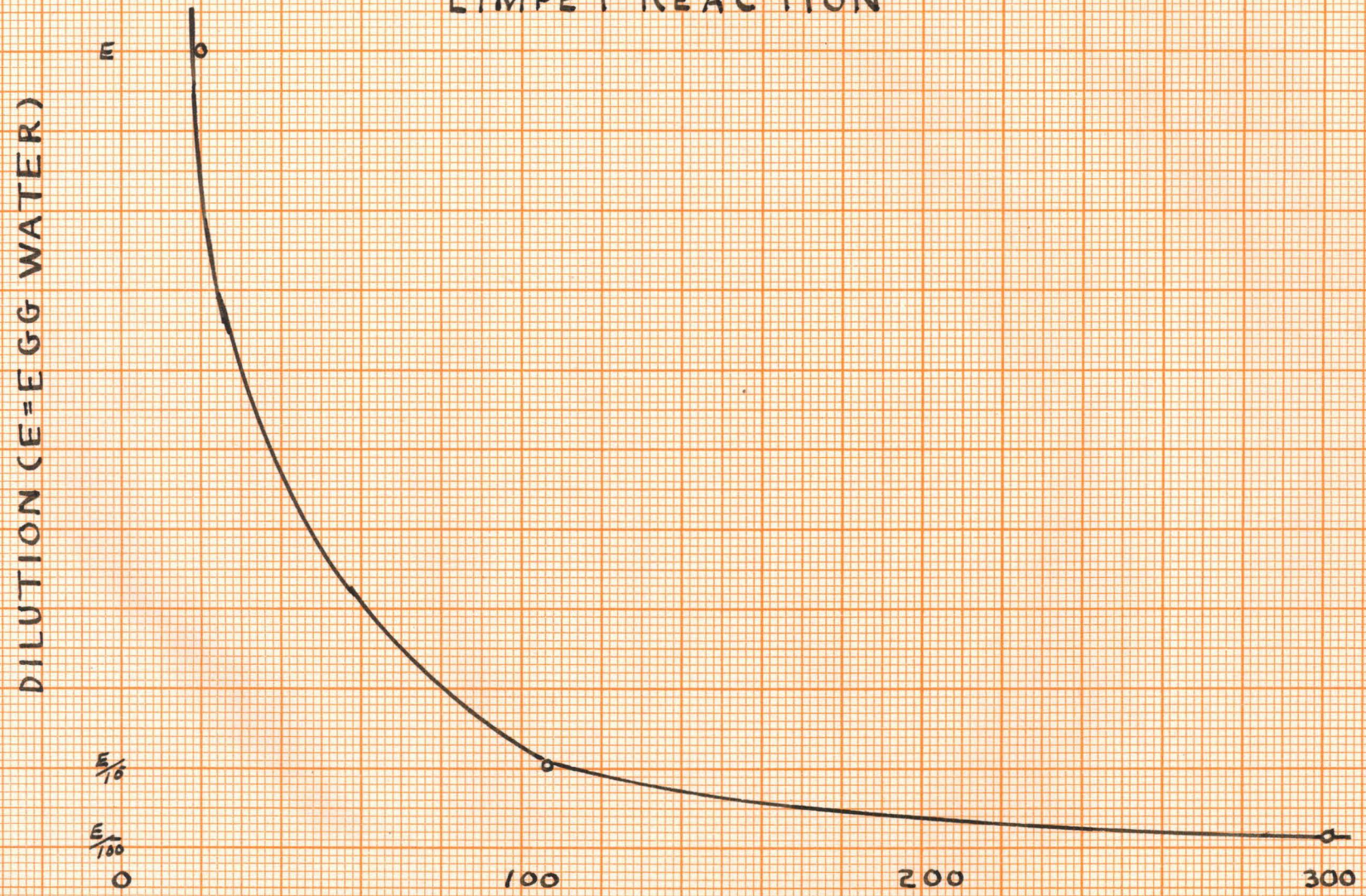
This more convenient and precise estimation consisted in determining the time at which clumps in a limpet reaction first became visible to the naked eye. The time of appearance showed its greatest sensitivity in the region of twenty seconds to three minutes. Stronger suspensions agglutinated more rapidly. In those cases in which the reaction appeared in less than twenty seconds it was found advisable to dilute the solutions in known proportions until the period exceeded the twenty-second value. In a series of tests run side by side, precision was about ± 4 seconds.

When 10 drops of sperm were swirled once with 10 drops of egg water of various dilutions, in Syracuse dishes, the following times were observed:

Concentration (E = Egg water)	Time for visible reaction (Seconds)
E	19
$\frac{E}{10}$	105
$\frac{E}{100}$	300
sea water	No reaction in 780

GRAPH ~~XVII~~

LIMPET REACTION



The Chemical Nature of Agglutinin

The most thorough study of the chemical nature of agglutinin had been carried out by Glaser (65) on Arbacia egg water. Of 14 tests which he applied, the only one which gave a weakly positive result was the xanthoproteic test. It was also reported by Woodward (66) that ammonium sulfate would precipitate an active material, containing nitrogen and carbon. The ability of Woodward to recognize agglutination has however been questioned (67). In review of all the evidence, Lillie was led to state subsequently that agglutinin does "not respond to the usual protein tests" (68).

Specificity of the reaction made it seem very likely that the agglutinin is a protein, or possibly a polysaccharide. It has often proved true that proteins present in sufficient concentration to invoke physiological responses exist in dilutions too high to respond to chemical color tests (69). Such a possibility seemed likely here.

Sea-urchin egg waters were concentrated by ultrafiltration through collodion membranes, and by evaporation of solvent. The residue from an ultrafiltration gave positive tests with Millon's Reagent, nitric acid, and biuret reagent, weak or negative reaction with lead acetate and alkali, and no response to p-dimethylaminobenzaldehyde. This indicated the

presence of protein containing tyrosine or homologs, possibly sulfur, and no tryptophane.

It was found that the limpet agglutinin could be conveniently concentrated from egg water by saturation with ammonium sulfate. This saturation had to be almost complete before any material would separate, indicating an albumin-like material. In repeated precipitations with ammonium sulfate followed by dialysis the first redissolved precipitate when allowed to stand 24 hours at 0°C., always left behind a portion of insoluble material (denatured protein?).

During experiments to prepare a concentrate of constant activity on successive reprecipitations with ammonium sulfate, it was found that a crude sea-water extract of limpet eggs retained approximately its original activity for two and one-half months when stored at 2-3°. Once the material was precipitated from sea water with ammonium sulfate and redissolved in sea water, however, its activity diminished appreciably in a few hours. It was therefore apparent that a reprecipitation-activity experiment would require working rapidly under conditions which would preserve the material.

Such an experiment was conducted by skimming off the precipitate from an ammonium sulfate solution, placing it on an ultrafilter and washing until the liquid was

sulfate-free, redissolving the residue from the filter and repeating the procedure. With one batch of egg-water this was carried out in a few hours.

The original egg water and the two successive precipitates were dialysed free of sea salts, and the non-diffusable material determined by evaporation of 10 cc. and weighing the residue in a tared weighing bottle on the micro balance. These concentration estimations were conducted subsequently to the estimation of activity. Since time for agglutination is not directly inversely proportional to concentration, the periods of agglutination were recorded for several concentrations of the purified second precipitate.

With this as an index the ratios of activities per milligram of dialysable material were found to be:

	Ratio
Egg-water	2.3
First precipitate	2.5
Second precipitate	1.0

A sample of the first precipitate was dialysed until free of salt. The resultant solution was evaporated in a desiccator over sulfuric acid. The residual material gave a Kjeldahl N of 2.8%. (The kindness of Mr. Willard McNary in performing the analysis is hereby acknowledged).

This same material gave a definite biuret test, and a positive xanthoproteic reaction. Tests for tryptophone and tyrosine in the molecule were negative.

Five milligrams of material were heated with 1 cc. of 1N HCl in a sealed tube at 100° for 3 hrs., but most of the solid did not dissolve. The procedure was repeated with another portion, concentrated HCl being employed this time. The solid dissolved and formed a brown solution. The solution was evaporated over steam, water added, and the operation repeated. The dark material, on addition of water, remained undissolved and was filtered off. The filtrate was neutralized to pH 7-8 with NaOH solution. A sample was then heated with Nylander's bismuth subnitrate solution to give a cloudy solution as compared to a control, but not the usual almost immediate darkening obtainable with reducing sugar. Upon examination a few days later, however, a darkened precipitate was seen to have separated.

It is evident then, in contrast to conclusions in the literature, that both sea-urchin and limpet fertilizin solutions contain protein. These specific proteins, furthermore, are distinguishable by different amino acid compositions, sea-urchin egg water protein containing the immunologically important hydroxyphenyl group, whereas limpet egg water protein does not. This protein accounts

for all or part of the nitrogen. The remaining material may be inert, and is probably glucidic. For an unequivocal conclusion as to the nature of the active moiety, the extract should be subjected to the action of pure protease and of pure glucidase or to procedures which will isolate one or the other in an active form.

One sample of limpet egg water was saturated with urea and stood at 28° for 48 hours. The urea was dialysed away and the dialysate was found to be as active as a control sample. This does not argue against the belief in a specific protein, since many proteins are not denatured by urea (70).

Further evidence for the complex nature of the substance is its heat lability, and lability to high pH and pOH as found by Tyler (71). The same investigator found that commercial trypsin would destroy the agglutinating activity of egg water.

It was also desirable to know whether an active material could be obtained by alcoholic precipitation. To this end a concentrated egg water was treated with 2 volumes of ethanol. The fibrous precipitate was removed and dried in a desiccator over sulfuric acid. The dried material was shaken with sea water, stood two hours, and filtered. The filtrate showed no agglutinating activity on limpet sperm in three minutes.

The yield of precipitate was however larger in contrast to the approximately five to fifty milligrams per liter of purified fertilizin ordinarily obtainable by the ammonium sulfate procedure.

Artificial Agglutinins

The finding of Carter (72) that thyroxine agglutinates the sperm of the English sea-urchins, Echinus miliaris and Echinus esculentus, led to trials of the same substance on the local sea-urchin.

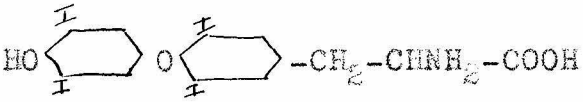
A study of the chemical specificity of thyroxine analogs in sperm agglutination might unearth correlations with immunology, especially inasmuch as Harington (73) has found thyroxine to be a powerful hapten when introduced onto protein by a modification of Bergmann's (74) carbobenzoxy method. The type of sperm clumping induced by thyroxine is irreversible.

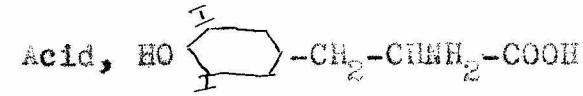
In the first attempt to find artificial agglutinins for sea-urchin sperm, sea water solutions of common proteins were employed. The following were tested:

Egg albumin solution
Casein "
Gelatin "

None of these when added in the ratio of 10 drops to 10 drops of a concentrated sperm suspension, produced agglutination.

The thyroxine analogs tried were:

Thyroxine, 

Iodogorgoic Acid, 

Tyrosine, 

Iodocasein,

Thyroglobulin.

The thyroxine was a Hoffman La-Roche solution (1:1000). It was diluted with an equal volume of "double" sea-water (sea-water concentrated to one-half its original volume).

The tyrosine was a stock material. One part was dissolved in 5000 of sea water.

Iodogorgoic acid was prepared by the method of Harington (75). Purification of this compound, often unsuccessful in other laboratories, was accomplished by rapid solution of the product in previously boiling water, and decanting the hot liquid from the brown resin which inevitably and invariably formed. White crystals then separated from the decanted liquor on cooling. Sea water was allowed to saturated with the compound.

Iodocasein resulted from the iodination of casein by the method of Liebricht (76). Sea water was allowed to saturate with the material.

Thyroglobulin was prepared by the method of Oswald (77)

from fresh thyroid glands. A solution containing 100 milligrams in 100 cc., with the pH adjusted to 8.3 by HCl and NaOH was employed.

In all cases 10 drops of each of the above were tested on 10 drops of a concentrated sea-urchin sperm suspension. The only solution which effected agglutination was that of thyroxine. Thyroxine, tyrosine, iodogorgoic acid, and a one percent solution of taurine in sea water were also tested on limpet sperm with no reaction.

Sperm Activation

In all egg waters studied, increased activity of sperm is known to occur in conjunction with agglutination. Some importance might be attached to this concomitance with relation to the role of agglutinin in fertilization. Clowes and Bachman (78) claimed that a number of organic substances would activate (increase the motility of) sperm. They were also able to obtain activation from the distillate of the egg waters of sea-urchin, sand-dollar, and starfish. They therefore suggested that the natural activator present is a mixture of autolytic products.

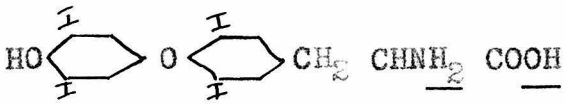
These results were substantiated and extended for the local sea-urchin. An egg water distillate diluted with an equal volume of "double" sea-water gave activation. The increase of motility of sperm in approximately 0.01 percent sea water solutions of two compounds previously undescribed in this connection, acetone and isopentanol, was very marked.

Conclusion

The conclusion derivable from the activation experiments is possibly ambiguous. While it is true that any one of many compounds present might be responsible for the activating effect of egg water, agglutinin might also be one of the many molecules which are capable of acting in this fashion. The fact that a number of types of organic compounds (ketones, unsaturates, alcohols) are stimulative makes it seem reasonable that a large molecule like the agglutinin possesses an essential functional group in itself.

The failure of thyroglobulin to cause agglutination of sea-urchin sperm is of special interest. In the thyroglobulin molecule the hydroxyl, iodo, and hydroxyphenyl groups of thyroxine are free, whereas the amino and carboxyl groups are coupled. It therefore seems likely from the above study that some combination of functional groups including the amino and/or carboxyl radicals of thyroxine are essential to sperm agglutination.

We may therefore provisionally underline in the thyroxine molecule these two groups as being of possible importance and leave the others in question:



A follow-up investigation would test various molecules

in which each of the groups in thyroxine is in turn masked, removed, or replaced.

Another suggestive correlation is to be found in the fact that on one hand thyroxine agglutinates sea-urchin sperm and not limpet sperm, while on the other hand egg water concentrates of sea-urchin give a positive Millon's reaction whereas the limpet concentrate does not. The Millon's reaction signifies the presence of tyrosine in a protein molecule, and indeed this amino acid, when coupled, is known to be of importance in immunology and specific agglutination phenomena. This correlation also adds circumstantial evidence to Carter's belief that the analogous amino acid, thyroxine, is related to sea-urchin agglutinin.

Although correlations in structure and activity evidently cannot be simply extrapolated from proteins to smaller molecules or vica versa, it is significant that active urchin egg extracts and an artificial agglutinin both contain the important hydroxyphenyl radical, whereas both of these conditions are totally absent in the limpet. This finding may thus be a first approach to the fundamental problem of chemical structure underlying biological specificity of the type exhibited by sperm agglutinins.

The isolation of sperm agglutinin, in summary, resulted in the preparation of labile concentrates which gave unquestionable protein and activity tests. The biological properties of agglutinins of various species were studied and bio-assays were developed. Evidence for typical behavior in protein concentration procedures and protein color tests has been presented. In conjunction with tests of lability to abnormal pH, heating, and tryptic action these indicate that the agglutinin is a protein or very closely associated therewith.

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