which reduces to

$$\left. \begin{aligned} V(\Delta G) &= k^2 \left[\frac{1}{m} + \frac{x^2}{S_1} + \frac{F_2^2(x)}{S_2} \right] \bar{T}^2 V(y_0), \\ \text{and similarly} \\ V(\Delta S) &= k^2 \left[\frac{1}{S_1} + \frac{4x^2}{S_2} \right] \frac{\bar{T}^2}{h^2} V(y_0), \\ V(\Delta H) &= k^2 \left[\frac{1}{m} + \frac{\bar{T}^2}{h^2 S_1} + \right. \\ &\quad \left. \frac{1}{S_2} \left(x^2 + \frac{2\bar{T}}{h} x + \frac{S_1}{m} \right)^2 \right] \bar{T}^2 V(y_0), \\ V(\Delta C_p) &= k^2 \frac{4}{h^4 S_2} (hx + \bar{T})^2 \bar{T}^2 V(y_0). \end{aligned} \right\} \quad (8)$$

It will be noted that the variances (8) depend on the temperature being used.

The discrepancy between $V(y_0)$ and $V(\log K)$ for set I of pK_a was found to be due to errors which, at given values of I , were correlated for the various

temperatures. Detailed analysis of the experimental records suggests that these correlated variations are due to small persistent differences in the silver-silver chloride electrodes. These errors account for part of the large value of $V(y_0)$ and, since they affect all temperature lines, would not greatly affect the values of y_i relative to one another. Hence, in these cases the fit of the quadratic equation (2) is better than would be expected.

It will be noted, therefore, that if $V(y_0)$ is inflated by correlated errors the various formulæ for variances will give considerable overestimates. It was found, however, that such situations rarely occurred.

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Thermodynamic Quantities for the Dissociation Equilibria of Biologically Important Compounds

2. THE ACID DISSOCIATIONS OF GLYCEROL 2-PHOSPHORIC ACID

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Glycerol 2-phosphate was chosen for first investigation because it is obtainable commercially and is readily purified. It is thus a convenient type substance for the important group of carbohydrate phosphate esters and a convenient compound for use in working out methods to be used with the scarcer materials.

EXPERIMENTAL

Purity of glycerol 2-phosphate. Although most commercial samples of disodium glycerol 2-phosphate are mixtures of the 1- and 2-isomers, the two can easily be separated. Thus Toal & Phillips (1949) showed that the first crop of crystals obtained from a 70% aqueous solution of a salt containing approximately equal proportions of the two isomers was predominantly the 2-form. It has been found in the present studies that preliminary crystallization from aqueous ethanol followed by crystallization from water, taking the first crop each time, gives substantially pure 2-isomer. It is essential to maintain the pH above 10 both to prevent the formation of acid salt and to obviate isomerization to the 1-ester which is catalysed by acid.

Disodium glycerol 2-phosphate (British Drug Houses Ltd.) was recrystallized three times from water:ethanol (75:25, v/v) and once from water. The crystals were washed with ice-cold water and dried over CaCl_2 . M.p. 100–102° (corr.). King & Pyman (1914) give m.p. 98–100°.

The product thus obtained analysed correctly for $\text{C}_3\text{H}_7\text{O}_6\text{Na}_2 \cdot 5\text{H}_2\text{O}$ by the following methods:

(i) Dry weight, obtained by heating to constant weight in an oven at 120°: calc., 70.57; found, 70.57, 70.58, 70.53%. (ii) Ignition to pyrophosphate of the dried salt in a platinum dish over a gas burner at bright-red heat until no further loss in weight occurred. Pyrophosphate as percentage of hydrated salt; calc., 43.43; found, 43.48, 43.38%. (iii) Paper chromatography of the ester (Hanes & Isherwood, 1949) was carried out using the modifications of Walker & Warren (1951). The papers were developed 28 hr. in *n*-propanol:ammonia:water containing ethylenediaminetetraacetic acid, which has been found to separate glycerol 1- and 2-phosphate. However, no orthophosphate, or glycerol 1-phosphate or other esters could be detected. Control experiments indicated that less than 1% of any could be detected by this method. Inorganic P was estimated by the method of Martin & Doty (1949) and was found to be 0.014% of the total P. Estimation of glycerol 1-phosphate by the method of Burmaster (1946) indicated the presence of 0.50% and this probably must be regarded as a maximum, since the acid present during the periodate oxidation may cause a small amount of isomerization.

The results of these experiments are in accord with the salt being highly purified disodium glycerol 2-phosphate. Its general properties agree in every way with descriptions in the literature of this salt (King & Pyman, 1914; Paolini, 1911, 1912; Bailly, 1916; Karrer & Salomon, 1926).

Dissociation constants. The experimental technique and methods of calculation were as described in Part I (Ashby, Crook & Datta, 1954). For the first dissociation, preliminary trials and then one set of experiments were done. A stock solution was prepared from which were made thirteen dilutions. The majority of these dilutions were measured in one cell from 5 to 30° and in another cell from 30 to 50°. The remainder were measured over one of these temperature ranges.

For the second dissociation, two sets of experiments were done using different stock solutions prepared from different samples of salt. In set I, eleven dilutions were prepared and each of these was measured over the full temperature range in a single filling of the cell. For set II, ten dilutions were prepared. These were measured in one cell from 5 to 25° and in another from 25 to 50°.

RESULTS

The results are set out in Tables 1–10. Table 1 contains the molalities of the solutions used for the first dissociation together with the observed e.m.f.'s corrected to one atmosphere pressure of hydrogen and a round temperature (i.e. to 5.00°, 10.00°, etc.). Table 2 contains similar data for the second dissociation.

From these are calculated the extrapolation functions (equations 10 and 15, paper 1), whose values are set out in Tables 3 and 4. Since the first dissociation is strong, the concentration of hydrogen ion is not only an important part of the ionic strength but also alters with the temperature. Thus, in Table 3 there is a separate value of the ionic strength for each dilution at each temperature. For the second dissociation, one value of the ionic strength suffices at all temperatures.

To the values of the extrapolation functions at each temperature the best straight lines were fitted by the method of least squares. The values at zero ionic strength were thus calculated. These are the thermodynamic pK 's. Their values are set out in Tables 5 and 6. The values of the thermodynamic pK 's were fitted to curves of the form

$$\log K = -A/T + D - CT$$

by means of orthogonal polynomials (Appendix above). The two sets of pK_2 values were fitted separately and combined into one final curve by averaging the constants of the polynomials. The calculated values at 37° and standard deviations are included in these tables.

The values of the constants A , D and C derived from these curves are listed in Table 7, together with the temperatures at which maximum ionization occurs (minimum pK) and the values of the pK 's at these temperatures.

From the values of the constants A , D and C the thermodynamic quantities for the ionizations are calculated by use of the appropriate relations (equations 17–22, paper 1). The quantities for the

first dissociation are set out in Table 8 and those for the second dissociation in Table 9. These are given in both joules and 15° calories in accordance with the recommendation of the Symbols Committee of the Royal Society (1951).

The values of the slopes of the extrapolation function (β) are given in Table 10. These will be useful in calculations involving the activity coefficient functions of these glycerol 2-phosphate: chloride mixtures.

DISCUSSION

The results presented here show that the methods for the determination of ionization constants and associated thermodynamic functions worked out by Harned and his collaborators are applicable to the type of phosphate ester which is of importance to biochemistry. While the present results do not exemplify the ultimate accuracy of which the method is capable, they do represent an advance on any data so far available in the literature for this type of compound.

Evidence has been presented to show that the samples of sodium glycerol 2-phosphate used in this investigation were substantially free from the 1-isomer. However, it is important to be sure that no isomerization occurred during the experiments. Bailly (1942) has shown that, even at 100°, no isomerization occurs above pH 2.15. The pK_2 determinations are thus certainly free from this effect. The pH of the solutions used for the determination of pK_1 was approximately 2.6 and, as the maximum temperature used was 50°, these would also appear to be free from any likelihood of conversion into the 1-form during the experiment. Tests conducted in this laboratory showed that at temperatures substantially below 100°, isomerization was not detectable even at pH 1.

No values for the thermodynamic quantities associated with the acid dissociations of glycerol 2-phosphate are to be found in the literature. It is, however, interesting to compare the present values with the data collected by Harned & Owen (1950) for a group of organic and inorganic acids. Such a comparison shows that the values for glycerol 2-phosphate fall within or near the range of values for the other acids. Glycerol 2-phosphate, however, has rather high values of $-\Delta C_p^0$, that for pK_2 being exceeded only by oxalic and malonic acids, and that for pK_1 heading the list of the known values. For many of these acids and, in particular, glycerol 2-phosphate, the change in heat content is in favour of the ionization. However, the entropy term is so large as to far outweigh the effect of the heat content change. Thus it appears to be the great decrease in entropy on ionization which causes these acids to be weak.

Table 1. *Glycerol 2-phosphate, 1st dissociation. Molalities of solutions and e.m.f.'s of cells; international volts corrected to 1 atmosphere pressure of hydrogen and rounded temperatures*

(m_1 = molality of HCl; m_2 = molality of disodium glycerol 2-phosphate. Two figures at 30° are given for each dilution. One was determined on one day following the determinations from 5 to 25°. The second was determined in a new filling of the cell on a subsequent day, using fresh electrodes, etc.)

	5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
m_1	0.090043	0.057945	0.39696	0.39773	0.39838	0.39900	0.39949	0.39946	0.40022	0.40071
m_2	0.048948	0.40365	0.40455	0.40526	0.40597	0.40650	0.40650	0.40649	0.40744	0.40808
	0.064479	0.41494	0.41027	0.41115	0.41192	0.41265	0.41336	0.41393	0.41440	0.41511
	0.050531	0.42015	0.42116	0.42208	0.42289	0.42360	0.42347	0.42417	0.42468	0.42520
	0.040816	0.42673	0.42905	0.43013	0.43210	0.43292	0.43269	0.43333	0.43395	0.43505
	0.031872	0.43781	0.44039	0.44153	0.44256	0.44349	—	—	—	—
	0.029950	—	—	—	—	—	0.44616	0.44708	0.44788	0.44913
	0.019992	0.45738	0.46055	0.46189	0.46316	0.46430	0.46430	0.46535	0.46629	0.46789
	0.015262	0.46891	0.47077	0.47247	0.47397	0.47540	0.47672	0.47780	0.47885	0.48074
	0.010629	—	—	—	—	—	—	0.49340	0.49481	0.49608
	0.010339	0.48654	0.48973	0.49147	0.49312	0.49467	—	—	—	—
	0.007758	—	—	—	—	—	0.50828	0.50983	0.51127	0.51266
	0.007641	0.49920	0.50140	0.50348	0.50541	0.50720	0.50889	0.51059	0.51207	0.51339
	—	—	—	—	—	—	—	—	—	0.51462

Table 2. *Glycerol 2-phosphate; 2nd dissociation. Molalities of solutions and e.m.f.'s of cells; international volts corrected to 1 atmosphere pressure of hydrogen and rounded temperatures*

(m_1 = molality of HCl; m_2 = molality of disodium glycerol 2-phosphate. In set II, two e.m.f.'s are given at 25°. This temperature is the break between successive days experiments. Set I was determined with no break in the measurements.)

		(a) Set I									
		5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
m_1	0.022936	0.042906	0.67604	0.68096	0.68590	0.69081	0.70070	0.70562	0.71057	0.71550	0.72054
	0.019888	0.037205	0.67950	0.68461	0.68943	0.69438	0.70460	0.70963	0.71472	0.71977	0.72480
	0.017683	0.033080	0.68218	0.68742	0.69246	0.69766	0.70798	0.71320	0.71830	0.72349	0.72873
	0.014068	0.026317	0.68908	0.69446	0.69957	0.70486	0.71006	0.72085	0.72606	0.73123	0.73651
	0.010263	0.019199	0.69789	0.70319	0.70862	0.71403	0.71953	0.73045	0.73591	0.74128	0.74676
	0.0092272	0.017262	0.70112	0.70652	0.71189	0.71717	0.72249	0.73340	0.73882	0.74461	0.75018
	0.0066498	0.012440	0.71001	0.71583	0.72142	0.72697	0.73260	0.74373	0.74937	0.75499	0.76083
	0.0045057	0.0084267	0.72077	0.72655	0.73226	0.73795	0.74371	0.75528	0.76113	0.76698	0.77285
	0.0033683	0.0072175	0.72475	0.73058	0.73633	0.74221	0.74810	0.75982	0.76566	0.77173	0.77781
	0.0019050	0.0035636	0.74350	0.74929	0.75562	0.76178	0.76794	0.78035	0.78659	0.79289	0.79925
	0.0016158	0.0030227	0.74771	0.75395	0.76013	0.76636	0.77270	0.78529	0.79161	0.79800	0.80431
		(b) Set II									
		5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
m_1	0.016324	0.033260	0.68922	0.69428	0.69940	0.70454	0.70974	0.71501	0.72024	0.72544	0.73069
	0.014907	0.030373	0.69179	0.69692	0.70210	0.70731	0.71257	0.71780	0.72308	0.72830	0.73362
	0.013000	0.026487	0.69560	0.70077	0.70597	0.71128	0.71657	0.72177	0.72725	0.73261	0.73797
	0.011260	0.022941	0.69970	0.70499	0.71026	0.71559	0.72091	0.72608	0.73168	0.73709	0.74245
	0.009056	0.018452	0.70579	0.71113	0.71654	0.72194	0.72753	0.73298	0.73854	0.74403	0.74969
	0.007379	0.015034	0.71123	0.71668	0.72222	0.72776	0.73340	0.73899	0.74463	0.75025	0.75597
	0.005874	0.011968	0.71753	0.72313	0.72875	0.73441	0.74018	0.74561	0.75099	0.75724	0.76302
	0.004256	0.008672	0.72620	0.73193	0.73766	0.74354	0.74945	0.75533	0.76126	0.76714	0.77313
	0.003957	0.008062	0.72809	0.73386	0.73964	0.74553	0.75142	—	—	—	—
	0.003865	0.007874	—	—	—	—	0.75210	0.75783	0.76384	0.76984	0.77597

Table 3. *Glycerol 2-phosphate, 1st dissociation. Values of ionic strengths (I) and extrapolation function (y) at rounded temperatures*

$$(y = \frac{(E - E_0)F}{2.3026RT} + \log \left(\frac{m_2}{m_1 - m_2 - m_H} - 1 \right) + \frac{2A/I}{1 + I})$$

Two sets of values appear for 30°, see legend to Table 1. For the first dissociation m_H is an appreciable fraction of the ionic strength and its variation with temperature appears as a variation in I .

5°		10°		15°		20°		25°		30°	
I	y	I	y	I	y	I	y	I	y	I	y
0.139565	1.1241	0.139080	1.1634	0.138601	1.2016	0.138106	1.2398	0.137530	1.2831	0.136984	1.3235
—	—	0.117853	1.1654	0.117420	1.2075	0.117032	1.2446	0.116544	1.2893	0.116120	1.3278
0.100810	1.1364	0.10047	1.1801	0.10019	1.2142	0.099885	1.2499	0.099516	1.2914	0.099164	1.3301
0.079440	1.1593	0.079242	1.1928	0.078934	1.2270	0.078793	1.2650	0.078544	1.3030	0.078280	1.3421
0.064455	1.1690	0.064251	1.2140	0.064116	1.2433	0.063925	1.2827	0.063730	1.3215	0.063529	1.3601
0.050624	1.1696	0.050507	1.2075	0.050418	1.2352	0.050293	1.2723	0.050153	1.3113	0.050011	1.3504
0.032027	1.1893	—	—	0.031913	1.2595	0.031864	1.2881	0.031795	1.3258	0.031727	1.3614
0.024556	1.1954	0.024508	1.2409	0.024469	1.2757	0.024438	1.3023	0.024393	1.3401	0.024342	1.3786
0.016718	1.2140	0.016703	1.2403	0.016695	1.2550	0.016680	1.2799	0.016657	1.3173	0.016629	1.3585
0.012394	1.2291	0.012389	1.2444	0.012384	1.2589	0.012377	1.2785	0.012368	1.3022	0.012356	1.3337
30°		35°		40°		45°		50°			
I	y	I	y	I	y	I	y	I	y		
0.137009	1.3218	0.136375	1.3675	0.135761	1.4112	0.135103	1.4573	0.134523	1.4980		
0.116126	1.3272	0.115606	1.3728	0.115052	1.4203	0.114525	1.4648	0.114005	1.5082		
0.099094	1.3373	0.098655	1.3837	0.098224	1.4282	0.097774	1.4737	0.097377	1.5136		
0.078946	1.3329	0.078001	1.3821	0.077703	1.4237	0.077336	1.4729	0.077012	1.5158		
0.063630	1.3419	0.063423	1.3810	0.063182	1.4249	0.062912	1.4721	0.062658	1.5155		
0.047096	1.3452	0.046913	1.3965	0.046737	1.4438	0.046563	1.4887	0.046411	1.5268		
0.031727	1.3614	0.031634	1.4072	0.031541	1.4508	0.031444	1.4939	0.031351	1.5338		
0.024366	1.3608	0.024299	1.4105	0.024240	1.4516	0.024163	1.5016	0.024105	1.5383		
0.017084	1.3641	0.017044	1.4169	0.017007	1.4601	0.016965	1.5069	0.016925	1.5493		
0.012531	1.3605	0.012511	1.4055	0.012489	1.4498	0.012458	1.5071	0.012431	1.5546		
0.012345	1.3593	0.012322	1.4106	0.012298	1.4597	0.012275	1.5037	0.012257	1.5366		

Table 4. *Glycerol 2-phosphate; 2nd dissociation. Values of ionic strengths (I) and extrapolation function (y) at rounded temperatures*

$(y = \frac{(E - E_0) F}{2.3026RT} + \log \frac{m_1^2}{m_2 - m_1} + \frac{2A\sqrt{I}}{1 + \sqrt{I}})$. Two sets of values appear for 25° in (b), see legend to Table 2. One value of *I* suffices for all temperatures since *m_H* is negligible.)

<i>I</i>	(a) Set I									
	5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
0.10578	6.6741	6.6695	6.6677	6.6687	6.6731	6.6802	6.6903	6.7028	6.7175	6.7344
0.09173	6.6621	6.6597	6.6546	6.6551	6.6612	6.6698	6.6805	6.6941	6.7095	6.7250
0.08156	6.6495	6.6484	6.6462	6.6500	6.6576	6.6643	6.6771	6.6898	6.7064	6.7242
0.06488	6.6564	6.6554	6.6521	6.6552	6.6596	6.6712	6.6831	6.6954	6.7095	6.7258
0.04733	6.6551	6.6498	6.6491	6.6514	6.6580	6.6670	6.6779	6.6915	6.7060	6.7227
0.04256	6.6598	6.6551	6.6523	6.6513	6.6539	6.6609	6.6719	6.6840	6.7043	6.7215
0.03067	6.6571	6.6568	6.6548	6.6554	6.6602	6.6662	6.6758	6.6885	6.7033	6.7219
0.02078	6.6604	6.6558	6.6524	6.6519	6.6556	6.6612	6.6718	6.6847	6.6998	6.7157
0.01779	6.6570	6.6520	6.6479	6.6494	6.6540	6.6597	6.6701	6.6815	6.6989	6.7169
0.008786	6.6588	6.6468	6.6469	6.6472	6.6504	6.6580	6.6663	6.6784	6.6938	6.7106
0.007452	6.6574	6.6520	6.6480	6.6480	6.6529	6.6581	6.6690	6.6811	6.6966	6.7112

<i>I</i>	(b) Set II									
	5°	10°	15°	20°	25°	30°	35°	40°	45°	50°
0.083456	6.6682	6.6617	6.6587	6.6595	6.6641	6.6680	6.6724	6.6835	6.6960	6.7286
0.076213	6.6676	6.6615	6.6587	6.6598	6.6646	6.6682	6.6713	6.6824	6.6944	6.7273
0.066461	6.6660	6.6593	6.6556	6.6571	6.6612	6.6632	6.6662	6.6794	6.6924	6.7237
0.057564	6.6666	6.6606	6.6567	6.6572	6.6605	6.6606	6.6636	6.6775	6.6901	6.7214
0.046298	6.6662	6.6590	6.6556	6.6553	6.6611	6.6608	6.6669	6.6781	6.6900	6.7236
0.037724	6.6617	6.6546	6.6516	6.6518	6.6567	6.6570	6.6631	6.6738	6.6861	6.7185
0.030029	6.6621	6.6555	6.6518	6.6520	6.6570	6.6565	6.6587	—	6.6839	6.7167
0.021760	6.6599	6.6527	6.6480	6.6492	6.6538	6.6538	6.6602	6.6709	6.6827	6.7152
0.020230	6.6590	6.6519	6.6475	6.6483	6.6519	—	—	—	—	—
0.019758	—	—	—	—	—	6.6519	6.6550	6.6663	6.6793	6.7137

Table 5. *Glycerol 2-phosphate; 1st dissociation. Observed and calculated values of thermodynamic pK at rounded temperatures*

(Calculated values are from $pK = A/T - D + CT$ where T is absolute temperature and A , D and C are constants fitted to observed values by the method of least squares.

σ , standard deviation of calculated values of pK_1 derived as explained in the Appendix to preceding paper = ± 0.005 .)

Temp. (°)	pK_1 (obs.)	pK_1 (calc.)
5	1.221	1.223
10	1.252	1.245
15	1.272	1.271
20	1.296	1.301
25	1.329	1.335
30	1.373	1.372
35	1.416	1.413
37	—	1.430
40	1.460	1.457
45	1.509	1.504
50	1.549	1.554

Table 6. *Glycerol 2-phosphate; 2nd dissociation. Observed and calculated values of thermodynamic pK at rounded temperatures*

(Calculated pK values from $pK = A/T - D + CT$ as in Table 5. Sets I and II were combined into a single equation by averaging the constants of the orthogonal polynomials used for the least squares fitting. σ , standard deviation of calculated value of pK_2 derived as in the Appendix to preceding paper = ± 0.0009 .)

Temp. (°)	pK_2 (obs.)			pK_2 (calc.)
	Set I	Set II	Set II	
5	6.656	6.657	—	6.657
10	6.650	6.650	—	6.650
15	6.647	6.645	—	6.646
20	6.647	6.646	—	6.646
25	6.651	6.650	6.650	6.650
30	6.657	—	6.653	6.657
35	6.667	—	6.667	6.666
37	—	—	—	6.671
40	6.679	—	6.677	6.679
45	6.695	—	6.693	6.694
50	6.712	—	6.710	6.712

Table 7. *Glycerol 2-phosphate; 1st and 2nd dissociations. Values of the constants of the equations relating thermodynamic pK and absolute temperature together with the minimum pK values and the Centigrade temperatures at which these occur*

Constant	Value for	
	pK_1	pK_2
A	1891.9072	1667.4043
D	13.4799	4.8394
C	0.02841	0.01978
$pK_{min.}$	1.182	6.646
$t_{min.}$	-13.2° C	17.25° C

For comparison with glycerol 2-phosphate the most interesting other acid is orthophosphoric. The most accurate data available for this acid are undoubtedly those of Nims (1934) and Bates (1951) for its pK_1 and those of Nims (1933) and of Bates & Acree (1943) for its pK_2 . The data at 25° are summarized in Table 11. Each pK of glycerol 2-phosphate is lower than the corresponding pK of orthophosphate, the difference being more pronounced at the first. The ΔG_i^0 values for glycerol 2-phosphate are correspondingly lower, as are those of ΔH_i^0 , but the values of $-\Delta S_i^0$ are very similar for both dissociations. The larger difference in pK at the first dissociation compared with that at the second is due both to a larger increase in $-\Delta H_i^0$ and to a smaller increase in $-\Delta S_i^0$ on passing from orthophosphoric to glycerol 2-phosphoric.

Perhaps the most striking difference between orthophosphoric and glycerol 2-phosphoric is that although both these acids show a trend of their pK 's with temperature similar to that shown by other acids in Harned & Owen's (1950) list, the minima in their second pK 's occur at widely different temperatures. Both pK_1 minima occur near -13°, but whereas the pK_2 minimum of orthophosphoric has risen to 44°, that for glycerol 2-phosphoric has only reached 17°. Both of these acids have lower pK_1 temperature minima than any other acid in Harned & Owen's list.

The accuracy of the present measurements can be estimated by the methods described in the Appendix above. The standard deviations thus calculated are included in Tables 5, 6, 8 and 9. These figures represent, of course, the reproducibility of the data, and do not allow for any bias introduced by the calibrations of the standard cell, resistance thermometer, weights, etc. Nor do they allow for possible differences between the values of E_0 and other fundamental constants used by us and by other observers. These will be small, and we have endeavoured to allow for them as much as possible by listing the values of those constants most subject to ambiguity which have been used in this work (Ashby *et al.* 1954). In addition there will be a small bias due to the particular extrapolation procedure used. This will be very small for pK_2 , but rather larger for pK_1 . At present there is no method of eliminating this as no extrapolation method is available which is free from ambiguity (Ashby *et al.* 1954).

The errors estimated here are of the same order as those suggested by Harned & Owen (1950, p. 516), although there is no indication as to how they arrived at their estimate since they do not appear to have calculated standard deviations. Harned & Owen suggest that the error in ΔH_i^0 is 50 cal. in the most favourable cases, and point out that it will be temperature-dependent, inasmuch as the estimates

Table 8. *Glycerol 2-phosphate; 1st dissociation. Thermodynamic quantities*

Temp. (°)	ΔG°		ΔH°		ΔS°		ΔC_p°	
	Cal.	Joule	Cal.	Joule	Cal. deg. ⁻¹	Joule deg. ⁻¹	Cal. deg. ⁻¹	Joule deg. ⁻¹
5	1556	6510	-1396	-5839	-10.6	-44.5	-72.3	-302.5
10	1612	6746	-1760	-7365	-11.9	-50.0	-73.6	-307.9
15	1675	7008	-2132	-8917	-13.2	-55.4	-74.9	-313.4
20	1745	7298	-2509	-10497	-14.5	-60.8	-76.2	-318.8
25	1820	7615	-2893	-12104	-15.8	-66.3	-77.5	-324.3
30	1903	7960	-3284	-13739	-17.1	-71.7	-78.8	-329.7
35	1992	8331	-3681	-15400	-18.4	-77.1	-80.1	-335.1
37	2029	8487	-3842	-16072	-19.0	-79.3	-80.6	-337.3
40	2087	8730	-4085	-17089	-19.7	-82.6	-81.4	-340.6
45	2189	9156	-4495	-18804	-21.0	-88.0	-82.7	-346.0
50	2297	9609	-4912	-20547	-22.3	-93.5	-84.0	-351.4

Errors

 $\sigma_5, \sigma_{25}, \sigma_{50}$ = standard errors at 5°, 25° and 50°, respectively.

σ_5	5.0	20.7	147	615	0.51	2.16	6.1	25.5
σ_{25}	3.0	12.5	43	179	0.15	0.63	6.5	27.2
σ_{50}	5.0	20.7	162	678	0.51	2.16	7.1	29.7

Table 9. *Glycerol 2-phosphate; 2nd dissociation. Thermodynamic quantities*

Temp. (°)	ΔG°		ΔH°		ΔS°		ΔC_p°	
	Cal.	Joule	Cal.	Joule	Cal. deg. ⁻¹	Joule deg. ⁻¹	Cal. deg. ⁻¹	Joule deg. ⁻¹
5	8469	35429	630	2637	-28.2	-117.9	-50.3	-210.5
10	8612	36028	376	1575	-29.1	-121.7	-51.2	-214.3
15	8760	36646	118	494	-30.0	-125.5	-52.1	-218.1
20	8912	37283	-145	-606	-30.9	-129.3	-53.0	-221.9
25	9069	37939	-412	-1725	-31.8	-133.1	-54.0	-225.7
30	9230	38614	-684	-2863	-32.7	-136.8	-54.9	-229.5
35	9396	39307	-961	-4020	-33.6	-140.6	-55.8	-233.3
37	9464	39590	-1073	-4488	-34.0	-142.1	-56.1	-234.8
40	9567	40020	-1242	-5195	-34.5	-144.4	-56.7	-237.0
45	9741	40752	-1528	-6390	-35.4	-148.2	-57.6	-240.8
50	9921	41502	-1818	-7604	-36.3	-152.0	-58.5	-244.6

Errors

 $\sigma_5, \sigma_{25}, \sigma_{50}$ = standard errors at 5°, 25° and 50°, respectively.

σ_5	1.0	4.0	29	120	0.10	0.41	1.17	4.89
σ_{25}	0.6	2.4	8	34	0.03	0.11	1.25	5.23
σ_{50}	1.0	4.0	31	130	0.10	0.41	1.36	5.69

Table 10. *Glycerol 2-phosphate. Values of 'β' (slope of extrapolation)*

(The columns labelled 'Graph' are values obtained by drawing a smooth curve through the experimental values by eye.)

Temp. (°)	pK_1		pK_2			
	Obs.	Graph	Set I		Set II	
			Obs.	Graph	Obs.	Graph
5	-0.765	-0.79	0.065	0.071	0.140	0.134
10	-0.692	-0.66	0.111	0.090	0.153	0.152
15	-0.533	-0.53	0.106	0.111	0.173	0.172
20	-0.410	-0.42	0.125	0.131	0.179	0.190
25	-0.318	-0.38	0.144	0.149	0.179, 0.203	0.208
30	-0.431	-0.37	0.168	0.166	0.226	0.225
35	-0.371	-0.37	0.183	0.182	0.234	0.238
40	-0.373	-0.38	0.191	0.191	0.236	0.237
45	-0.402	-0.39	0.184	0.187	0.223	0.226
50	-0.386	-0.40	0.185	0.180	0.216	0.212

at the two ends of the temperature range will be less reliable than those near the middle. It will be seen that the accuracy of ΔH_i° for pK_2 is rather better than this, especially at the centre of the range. The error is larger for pK_1 , but must be judged satisfactory since this is not so favourable a case. The errors in ΔG_i° and ΔS_i° are symmetrical about the middle of the temperature range, but the error in ΔH_i° is symmetrical about a point somewhat displaced (27.2°) from the middle (27.5°). For $\Delta C_{p,i}^\circ$ there is no minimum, the error increasing directly as the absolute temperature (Appendix above). It seems incorrect, therefore, to conclude, as Harned & Owen (1950, p. 516) appear to have done, that the values of $\Delta C_{p,i}^\circ$ are most accurate at the middle of the temperature range. In fact, the low-temperature values have the smallest uncertainty. However, the magnitude of the error they suggest is approximately the same as that observed here: $\pm 1 \text{ cal. deg.}^{-1} \text{ mole}^{-1}$ for the best values (pK_2) and up to $\pm 7 \text{ cal.}$ in the more unfavourable cases.

The only values for the dissociation constants of glycerol 2-phosphate in the literature are the apparent pK 's summarized in Table 12. It is impossible to make an accurate comparison of these figures with the present data since the conditions under which they were obtained are not all known

Table 11. *First and second thermodynamic pK values of orthophosphate and of glycerol 2-phosphate at 25°*

	pK_1	pK_2
Phosphate		
Nimms (1933, 1934)	2.123	7.206
Bates & Acree (1943)	—	7.198
Bates (1951)	2.148	—
Glycerol 2-phosphate		
(This paper)	1.335	6.650

with accuracy. Moreover, the present values are stated on the molal scale. Conversion from one scale into the other can be carried out by means of the correction factors listed in Table 2 of Paper 1. However, for most purposes the differences in pK are too small to be important except at high temperatures. Activity coefficients decrease rapidly above an ionic strength of 0.01, particularly for the doubly charged ions resulting from second dissociations. Differences between thermodynamic and apparent pK 's may therefore amount to 0.5 pH unit or more. However, an estimate of the thermodynamic pK at various concentrations may be obtained from the apparent pK by using the Guntelberg equation to calculate the activity coefficients (Ashby *et al.* 1954). The pK values in Table 12 thus calculated for the conditions of measurement of the various workers show a reasonable agreement with the present measurements.

However, the errors involved in trying to make such comparisons show how misleading it may be to draw conclusions about the relative strengths of acids from apparent pK 's determined under unspecified conditions. The errors may exceed 0.3 pH unit, whereas decisions about inductive effects and those due to solvation, etc., often turn on differences of 0.1 unit or less. Thus, the conclusions of Kumler & Eiler (1943) about the relative strengths of glycerol 1- and 2-phosphate may be seriously in error since they depend on differences of 0.03 in the pK_1 's and 0.1 in the pK_2 's.

Apparent pK values are comparable only when determined under strictly standardized conditions and even then are subject to errors in interpretation. The thermodynamic pK 's, provide a much better basis for comparison between acids and investigations of the effects of structure and other factors on the ionization should be based upon these values

Table 12. *Thermodynamic pK 's of glycerol 2-phosphate calculated from literature values of the apparent pK 's*

(Abbreviations: R.T. = room temperature; I = ionic strength; pK' = apparent constant; pK = thermodynamic constant. Thermodynamic pK_2 's were calculated from apparent constants by: $pK = pK' + \frac{3A\sqrt{I}}{1 + \sqrt{I}}$, where I = ionic strength at which pK' was determined; where possible, the value at or near maximum buffering. A is the Debye-Hückel constant. Apparent pK_1 's were calculated by all authors assuming $pH = -\log m_H$ and calculating $K' = \frac{m_H(m_{\text{salt}} + m_H)}{(m_{\text{acid}} - m_H)}$. Neglect of the activity coefficient in both cancels and the calculated pK' is close to the calculated pK obtained by introducing the activities calculated by the Guntelberg equation (Ashby, Crook & Datta, 1954).)

Author	Electrode	Temp.	First dissociation			Second dissociation		
			I	pK_1'	pK_1	I	pK_2'	pK_2
Meyerhof & Suranyi (1926)	H ₂	20°	0.051	1.40	1.40	0.073	6.30	6.62
Morton (1930)	Glass	18°	0.062	1.36	1.35	0.090	6.29	6.64
Kiessling (1934)	H ₂	R.T.	0.063	1.37	1.37	0.080	6.34	6.67
King & Delory (1939)	H ₂	R.T.	—	—	—	0.024	6.34	6.54
Delory & King (1943)								
This paper	H ₂	20°	—	—	1.301	—	—	6.646

(Dippy, 1939). However, these are not available for most of the biologically interesting compounds. Even the thermodynamic pK 's are not entirely free from ambiguity because the ionization constants of different compounds change with temperature at different rates and the relative strengths of a group of acids alter with temperature. No completely satisfactory basis of comparison has yet been suggested.

An important aspect of the study of the thermodynamics of the ionization of phosphate esters is that it allows the calculation of the proportion of the free energy of hydrolysis of the ester which is accounted for by changes in the ionization under various conditions. For the hydrolysis of the free acid forms of phosphate esters, or of self-buffering mixtures of their salts and acids, there will be negligible change in the state of ionization on hydrolysis at a pH above 3. The pH will change due to the alteration of pK from that of the ester to that of orthophosphate, but the total concentration of H^+ ion is so small that a negligible alteration in the ratio of salt to acid forms suffices to bring about the necessary adjustment. Under these conditions, therefore, the free energy of hydrolysis will not contain a term due to ionization changes.

However, when the pH of the medium is maintained substantially constant, as it may be in the cell, then substantial changes in ionization occur. We find, in agreement with Hill & Morales (1951) and in disagreement with Oesper (1950), that the change in free energy on ionization into a medium of constant pH is given by

$$\Delta G = 2.3026RT \log [m_H/(K + m_H)]$$

to a first approximation. The change in free energy during the hydrolysis of a phosphate ester in such a medium due to changes in the ionization will be given by the difference between two such expressions. Thus, the change in ionization when one mole of glycerol 2-phosphate is completely hydrolysed in solution at pH 7.4 and 37° corresponds to a change in free energy of nearly -200 cal. As the overall $-\Delta G^\circ$ of hydrolysis is approximately 2200 cal., the energy of ionization is seen to amount to nearly 10% of the total. The change would be proportionally less had the free energy of hydrolysis been larger, but even at 11000 cal. mole⁻¹ would account for 2% of the total in compounds with a pK similar to that of glycerol 2-phosphate.

In conclusion, it is worth noting that sufficient information has now been given about the acid dissociations of glycerol 2-phosphate in the presence of sodium chloride to allow these mixtures to be used as standard buffers over the pH range 5-7 and at ionic strengths up to 0.15. While the data are not as accurate as those of Bates & Acree (1943) for phosphate, they may on occasion be useful.

SUMMARY

1. The first and second thermodynamic dissociation constants of glycerol 2-phosphate on the molal scale have been measured at 5° intervals from 5 to 50° using hydrogen and silver-silver chloride electrodes in cells without liquid junctions.

2. From these measurements have been calculated the standard free energy, heat content, entropy and heat capacity for the two ionizations over the whole temperature range, together with the respective standard deviations of the values obtained.

3. The magnitude and sources of the errors are discussed.

4. Some assessment is given of the importance of changes in ionization during the hydrolysis of phosphate esters and of the proportion of the total free-energy change assignable to this change.

5. The results demonstrate that the methods worked out by Harned & Owen (1950) for inorganic acids are applicable to compounds of interest to biochemists and also that the glycerol phosphoric acids closely resemble the simpler weak acids in their dissociation properties.

6. Sufficient information has been given about the acid dissociations of glycerol 2-phosphate to allow its solutions to be used as standard buffers in the pH range 5-7 and at ionic strengths up to 0.15.

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Micro-estimation of 'Formaldehydogenic Steroids' by Bismuthate Oxidation

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Oxidation of the C-17 steroid side chain,

to formaldehyde forms the chemical basis for the quantitative determination of the so-called 'formaldehydogenic steroids'. The method can be considered in three stages—the oxidation, the transfer of formaldehyde, the colorimetric determination of formaldehyde—and the completeness of each stage may be examined independently. In current methods the oxidation is carried out at room temperature by periodic acid, excess of this reagent being removed by stannous chloride (Lowenstein, Corcoran & Page, 1946; Corcoran & Page, 1948). Although in early methods the formaldehyde was estimated in the oxidized solution, it is advantageous to isolate the formaldehyde by distillation before assay (Daughaday, Jaffe & Williams, 1948). This has been carried out conveniently, on a micro scale, by isothermal distillation in Conway units (Bassil & Hain, 1950; Hollander, Di Mauro & Pearson, 1951). The formaldehyde produced is estimated by heating with chromotropic acid reagent (MacFadyen, 1945).

Whilst present methods of assay are sufficiently reliable for most clinical purposes, the published methods, even when applied to pure compounds, lack precision. Stage by stage examination showed that whilst the determination of formaldehyde was quantitative, the recovery of formaldehyde by isothermal distillation in conventional Conway units was neither complete nor reproducible. When periodate was used as the oxidant, the high blanks encountered limited the usefulness of the method at

low concentrations. In the present method, sodium bismuthate (Rigby, 1950) replaces periodic acid as the glycol fission agent and is subsequently removed by centrifuging; the formaldehyde is determined after isothermal distillation in a modified Conway unit. This unit permits the transfer of the formaldehyde directly into the chromotropic acid reagent at 100°, the transfer and simultaneous colour development being complete in half an hour. The procedure is applicable in the range of 1–10 µg. of steroid (accuracy ± 0.05 µg.).

The use of bismuthate for the oxidation of corticosteroids has been suggested independently by Brooks & Norymberski (1952) as a basis for the determination of formaldehydogenic steroids and of 17-ketogenic steroids. By studying the action of this reagent upon compounds representing the different types of C-17 side chain encountered in corticosteroids these workers have demonstrated the value of such methods for the determination of corticosteroid metabolites in urine (Brooks & Norymberski, 1953; Norymberski, Stubbs & West, 1953).

EXPERIMENTAL

Recovery of formaldehyde by isothermal distillation

The formaldehyde solution (100 µl. containing 0.5 µg.), prepared by dilution from distilled formalin, was placed in the outer compartment of a no. 2 Conway unit (Conway, 1947), and isothermally distilled into the chromotropic acid reagent (400 µl.) in the inner compartment. At the end of the distillation the contents of the inner compartment were transferred to a small test tube and heated at 100° for 30 min. The percentage recovery figures (Table 1) were based upon the colour density so obtained, taking as 100% that recovery given by heating the same proportions of chromotropic acid reagent and formaldehyde for the same period of time after direct mixing.

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