THE IN VITRO OXIDATION OF TESTOSTERONE

BY GUINEA PIG LIVER

APPROVED BY

DISSERTATION COMMITTEE

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THE IN VITRO OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

CHAPTER I

INTRODUCTION

Knowledge concerning the chemistry and the specific physiological effects of the steroid hormones has progressed rapidly since the isolation of estrone in 1929. Since that time the basic steroid structure has been established, and many naturally occurring steroids have been isolated from a number of tissues and from urine. Most of these naturally occurring steroids have been structurally identified, and some have been synthesized along with many others with related structures which do not naturally occur.

The metabolic synthesis and degradation of many of the naturally occurring steroids have been studied by *in vivo* and *in vitro* methods in human beings as well as in experimental animals. The physiological effects of administration of both natural and synthetic steroids have been determined on a number of species and the effects which these steroids may have on the processes of life seem to broaden as more investigations are made.

A number of treatises (1, 2) and monographs (3, 4, 5) and a symposium (6) have appeared, along with annual publications (7, 8) and

several review articles (9, 10, 11) which deal with both specific and general topics in steroid chemistry and physiology. These general references serve as an introduction to the vast field of steroid endocrinology, and other literature cited will be limited to that which is directly concerned with the studies described in this dissertation.

The administration of adequate amounts of testosterone to experimental animals or human beings results in a distinct increment in the quantity of androgens and 17-keto steroids excreted in the urine. The increment is dependent on the amount administered. Observations have been made in the human (12, 13, 14, 15) and studies have also been reported for the guinea pig (16, 17), monkey (18, 19) and chimpanzee (20). The principle androgenic metabolite excreted in man was shown to be androsterone (21, 22) with etiocholan-3α-ol-17-one (21, 23, 24) and epiandrosterone (25) in lesser amounts. In the guinea pig the administration of testosterone gives rise to etiocholan-3α-ol-17-one and epiandrosterone (17, 26) in the urine. It appears that the guinea pig favors production of 3β-hydroxy ketosteroids, whereas man, monkey and the chimpanzee favor the 3α-hydroxy compounds.

In vitro studies by Clark and Kochakian (27) have shown that incubation of testosterone with rabbit liver slices produced 4-androstene-3,17-dione and epitestosterone. This reaction was reversible, as incubation of 4-androstene-3,17-dione with rabbit liver slices gave rise to testosterone as well as epitestosterone (28). The same conversion was effected with rabbit liver homogenates (29).

The involvement of cofactors in enzyme reactions was suspected as early as 1893 (30) and 1906 (31), but no pure nicotinamide coenzyme

became available until 1935 when diphosphopyridine nucleotide (DPN) was isolated and identified by Schlenk and V. Euler (32). Warburg and Christian (33) are credited with the discovery of triphosphopyridine nucleotide (TPN). Since then these coenzymes have been associated with a number of oxidation-reduction reactions. Samuels (34) demonstrated that rat liver minces destroyed testosterone and that the rate of destruction could be enhanced by coenzyme I (DPN) (35). Kochakian, et al., (36) observed that the incubation of 4-androstene-3,17-dione with guinea pig liver and kidney brei yielded testosterone and androsterone. Testosterone incubated with guinea pig liver homogenates was oxidized to 4-androstene-3,17-dione. The presence of diphosphopyridine nucleotide increased the yield still further.

These experiments with guinea pig tissues were based on the total conversion of the substrate to products in experiments designed to allow the reaction to come to equilibrium, at which time the reaction products were determined qualitatively and quantitatively and the percentage conversions were determined. This type of experiment yields invaluable information but gives no information as to the rate of the reaction involved and thus is not a measurement of amounts of enzyme present. Hamm (37), in initial experiments patterned from the equilibrium experiments, demonstrated that DPN was reduced (DPNH) as evidenced spectophotometrically when guinea pig liver homogenates were incubated with a water solution of testosterone. He also demonstrated that TPN was also reduced (TPNH) under the same conditions, indicating a dual capability of guinea pig liver to oxidize testosterone by using either DPN or TPN as a coenzyme. Measurement of rates of reaction involving these pyridine nucleotide

coenzymes is facilitated by the fact that these molecules have an absorption at 2600 Å in the oxidized form (TPN or DPN), but in the reduced form (TPNH or DPNH) they also have an absorption at 3400 Å which is proportional to the concentration of the reduced form. No attempt was made, however, to demonstrate that the rate of DPNH formation correlated with the rate of metabolism of testosterone.

Despite the rapid increase in knowledge concerning the chemistry and the physiological effects of steroids, and the androgens in particular, the question of how these compounds with a nucleus related to 1,2-cyclopentanoperhydrophenanthrene manifest their effects is still unanswered. Testosterone, the most active natural androgen in man, is known to be metabolized and excreted primarily to androsterone and etiocholan-3α-ol-17-one in man and to the 3β-hydroxy derivatives in the guinea pig. The primary site seems to be in the liver, although metabolism occurs in other tissues. Whether this metabolism is a necessary set of reactions for the manifestation of the physiological effects of testosterone or is merely a means of excreting the material after the physiological effect takes place is not known.

Experiments by Biskind and Mark (38) indicate that with respect to testosterone the liver is primarily a site of inactivation. They demonstrated that the implantation of pellets of testosterone or of methyl testosterone in the spleen of immature or castrated rats did not lead to precocious development of the secondary sex organs as long as the spleen was left in situ. If, however, the spleen was transplanted so that the blood drained into the general circulation, the seminal vesicles

and prostate immediately underwent great enlargement.

It would seem that a further knowledge of the reactions concerned with the step by step metabolism of testosterone would yield valuable information which might be used to determine its mode of action physiologically.

In order to study the individual reactions involved in the metabolism of testosterone, it is necessary to work with isolated enzyme systems. This method, although heavily criticized in previous years as being "unphysiological," has been the method whereby many of the processes of metabolism, especially those leading to the production and utilization of energy, have been clarified and established.

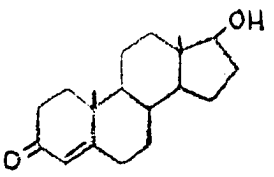
Hamm (37) started the studies on the metabolism of testosterone by isolated enzyme systems by demonstrating that the cofactor duplicity mentioned above could be resolved by centrifugation of the homogenate of guinea pig liver, the DPN-specific activity being associated with the mitochondrial fraction while the TPN specific reaction was associated with the soluble fraction at 24,500 X G.

This presented the problem of whether two enzymes were associated with these activities rather than a single enzyme capable of using two cofactors, or, if indeed it was possible the activity involving two cofactors was only an apparent activity with the hydrogen being transferred from one cofactor to the other by means of a transferase. This latter idea has been suggested by Talalay and William-Ashman (39) with the estrogen stimulated DPN specific isocitric dehydrogenase demonstrated by Villee and Gordon (40, 41, 42). The purpose of this dissertation

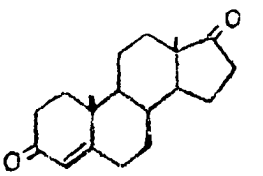
is to contribute information towards the solution of this problem. A systematic nomenclature of androgens has been devised (43) but many trivial names are still in use today. The systematic nomenclature as well as the trivial names for the steroids used in this dissertation are given in Figure 1.

FIGURE 1

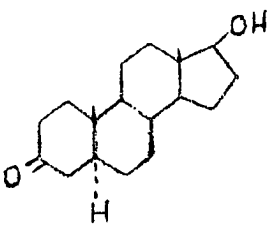
STEROID STRUCTURE



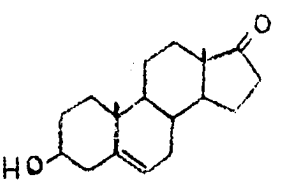
4-androsten-17β-ol-3-one
(testosterone)



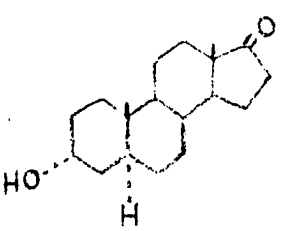
4-androstene-3,17-dione
(androstenedione)



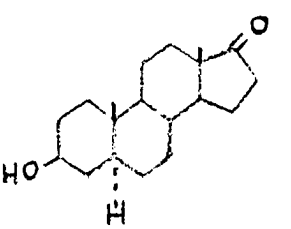
androstan-17β-ol-3-one
(androstanolone)



5-androsten-3β-ol-17-one
(dehydroepiandrosterone)

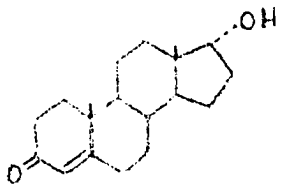


androstan-3α-ol-17-one
(androsterone)

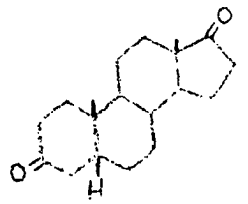


androstan-3β-ol-17-one
(epiandrosterone)

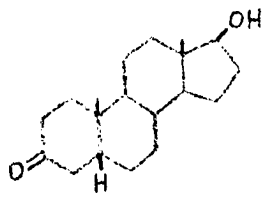
FIGURE 1
(continued)



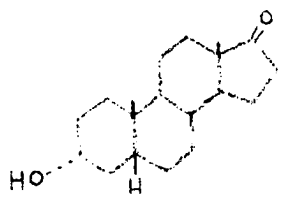
4-androsten-17α-ol-3-one
(epitestosterone)



etiocholane-3,17-dione



etiocholan-17β-ol-3-one



etiocholan-3α-ol-17-one

CHAPTER II

GENERAL EXPERIMENTAL METHODS

Enzyme Source

Animals

Normal male guinea pigs were used as a source of tissue during the course of these experiments. These animals were purchased at 500-600 grams body weight. They were fed commercial rabbit pellets *ad libitum* with 50 mg Vitamin C supplement each week and kept in a constant temperature room at 72°F. They were maintained two per cage and weighed twice weekly on a Shadowgraph balance.

Preparation of Tissue

These animals were sacrificed at approximately 1000 grams body weight by a blow on the head followed by exsanguination by severing the blood vessels in the neck region. The tissue to be used (liver) was removed quickly and as carefully as possible to prevent excess contamination by bacteria and hair and then cooled immediately in a beaker in a bath of crushed ice. The tissue was homogenized shortly thereafter (within 10-20 minutes) at a concentration of either 10 or 25 percent wet weight.

Preliminary Fractionation Procedures

The fractionation of guinea pig liver homogenate for use in the preliminary characterization of the DPN or TPN specific reactions was done in a manner similar to that described by Hogeboom for rat liver (44). The following fractionation procedure was adhered to for preparation of fractions and during the experiments of Chapters III and IV.

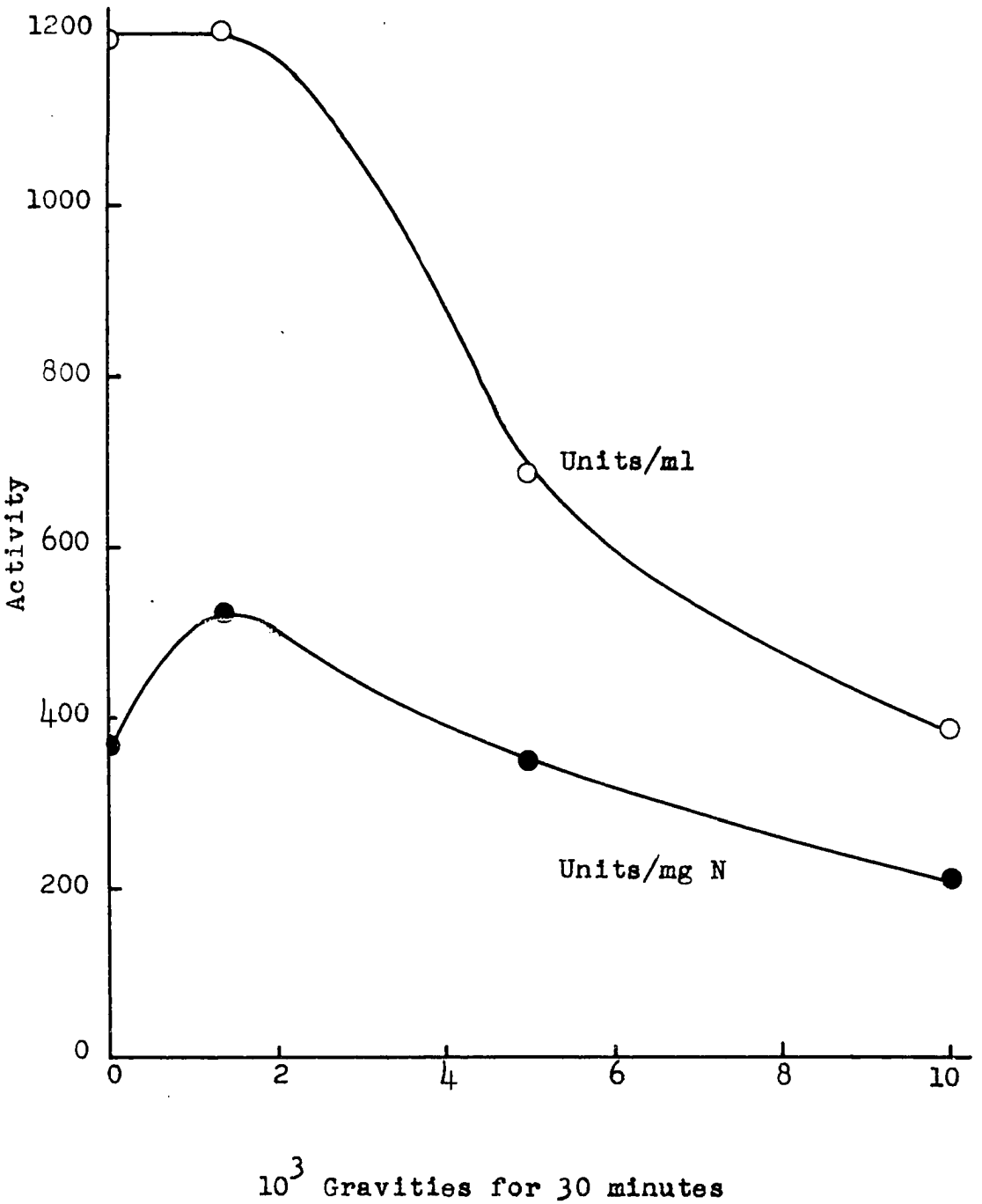
Fresh guinea pig liver was homogenized in a Virtis, Model No. 45, homogenizer at a concentration of 10 grams/100 ml of 0.25 M sucrose. Homogenization was carried out in the 250 ml homogenization flask of the Virtis set in an ice bath for 1 minute with dial set at 30 and for 15 seconds at 50. The total volume ranged from 100 to 200 ml. A portion of the homogenate was removed for enzyme and nitrogen assay. A given known amount was then centrifuged at 1500 X G for 30 minutes.

A preliminary experiment was run in which the homogenate was centrifuged for 30 minutes at 0, 1400, 5000 and 10,000 X G, and the enzyme activities using DPN as coenzyme and nitrogen content of the supernatant were compared. It demonstrated that no enzyme activity using DPN as the coenzyme was lost from the supernatant at 1500 X G for 30 minutes (Figure 2). Therefore the sediment from 1500 X G was discarded except in balance sheet experiments, in which case it was diluted up to the original volume.

The supernatant from 1500 X G was centrifuged at 24,500 X G for 1 hour, after which time the supernatant was decanted off and the sediment was resuspended in the original volume of cold 0.25 M sucrose. This sediment is called SED_2 or mitochondrial fraction throughout this

FIGURE 2

THE EFFECT OF CENTRIFUGATION AT LOW G. ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE



dissertation. The mitochondrial fraction was used as the enzyme source for experiments on the oxidation of testesterone in which DPN was used as coenzyme, and supernatant 24,500 X G was used as enzyme source for the experiments in which TPN was used as coenzyme.

Two different methods of homogenization of liver were compared with respect to their effect on the rate of conversion of testosterone to 4-androstene-3,17-dione. These methods were homogenization in glass Erway homogenizers and homogenization in a Virtis homogenizer. Since there was no significant difference in the rate of oxidation of testosterone using tissue prepared by these methods (Table 1), the Erway homogenizer was used when small amounts of tissue homogenate were needed, and the Virtis was utilized when large amounts were needed as in the case of the fractionation experiments. Because of the possibility of shearing mitochondria, the Virtis homogenizer was used only for short periods of 1 to 2 minutes.

Two different media were also utilized for the homogenizing media. These media were water and 0.25 M sucrose. Only slight differences were noted in the activity of the homogenate in either medium (Table 1), but because the tissue fractionated best in 0.25 M sucrose (Figures 25 and 26) this medium was usually employed.

A schematic fractionation of a typical guinea pig liver preparation is illustrated in Figure 3. A tabulated list of all guinea pig preparations used in this dissertation listing the enzyme activity of the DPN and TPN linked enzymes of all fractions on a wet weight and also on a mg nitrogen basis is given in Table 2.

TABLE 1

COMPARISON OF HOMOGENIZATION IN GLASS AND VIRTIS AND SUCROSE AND WATER

Means	Medium	mg N per ml	Units Activity*			
			DPN		TPN	
			per ml	per mg N	per ml	per mg N
Virtis	Sucrose	3.32	1125	339	442	133
Virtis	Water	3.29	986	300	404	123
Glass (Erway)	Sucrose	3.50	1070	306	426	122
Glass (Erway)	Water	3.48	926	266	414	119

*A unit of activity equals the change in OD of 0.001/min.

Method of Measurement of Enzyme Activity

Rate Measurement

A Cary recording spectrophotometer Model 14, equipped with a cell compartment which could be thermostatically controlled was used to measure reaction rates. The Cary spectrophotometer is so designed that it records the difference in absorption at any wave length between a reference cell and the sample cell at any time. Thus, with the proper design of reactants in the reference cell compared with the experimental cell, the endogenous activity (or reactions) is automatically subtracted from the experimental reaction being studied. Thus, in measuring the rate of oxidation of substrate (testosterone or other steroids) by guinea pig liver fractions, the same reaction components were put in the reference cell as in the experimental cell with the exception of the substrate.

FIGURE 3

SCHEMATIC FRACTIONATION OF TISSUE

Homogenization at 10 per cent in Virtis
in 0.25 M Sucrose

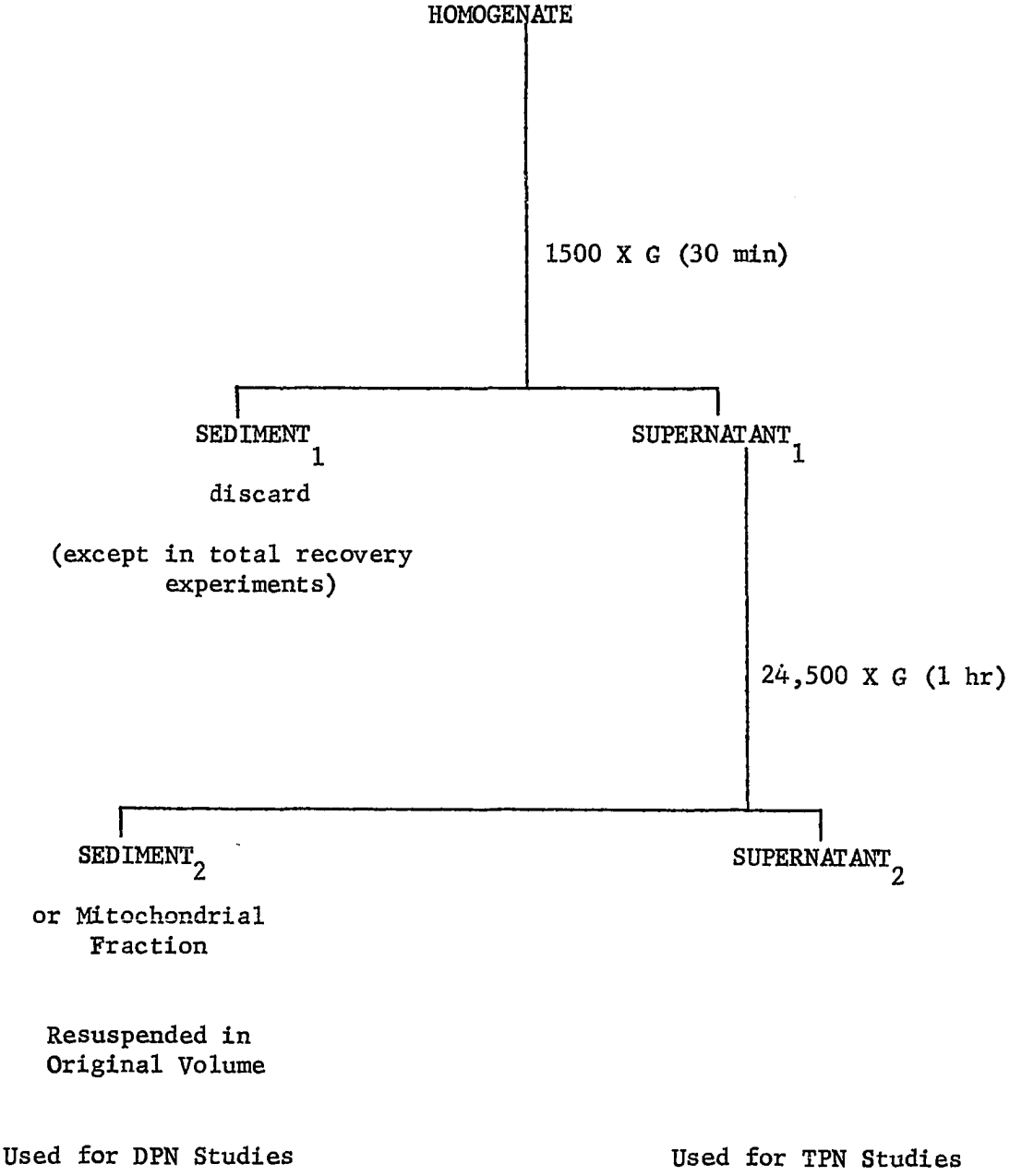


TABLE 2

GUINEA PIG LIVER ENZYME PREPARATIONS

Prep #	Homogenate				
		DPN		TPN	
		Activity[a] Units			
	mg N per ml	per ml	per mg N	per ml	per mg N
1	3.26	319	98		
2[b]	3.37	471	140	323	96
3[c]	3.85	390	101		
4	3.27	604	184	507	155
5	2.45[d]	621	253	314	129
6[e]	3.38	1478	437	707	209
7	3.14	896	286	160	51
8	3.50	1530	437		
9	3.37	1240	368	493	146
10		1440		447	
11	3.22	1200	372		
12	3.48	1367	393	580	167
13	3.10	917	296		
14	3.24	1147	354		
15	3.80	1265	333	650	171
16	3.29				

TABLE 2, continued

Sediment 24,500 X G			Supernatant 24,500 X G		
	DPN			TPN	
	Activity[a] Units			Activity[a] Units	
mg N per ml	per ml	per mg N	mg N per ml	per ml	per mg N
0.70	274	391	1.47	173	117
0.89			1.66	200	120
0.89	228	256	1.72	203	118
0.89	490	554	1.73	337	194
0.93	490	525	1.56	198	127
0.82	1093	1330	1.67	724	434
0.83			1.56		
0.85	620	729	1.70	462	272
0.84	586	698	1.65	439	266
	636			443	
0.88	543	617	1.59	497	312
0.64	597	933			
0.76			1.78	386	217
0.85	1131	1330	1.80		

TABLE 2, continued

GUINEA PIG LIVER ENZYME PREPARATIONS

Prep #	Homogenate				
		DPN		TPN	
		Activity[a] Units			
	mg N per ml	per ml	per mg N	per ml	per mg N
17	2.90	1350	466	635	220
18	2.21[f]	1040	471	541	245
19	2.32[f]			435	188
20	2.96	1201	406	703	238
21	2.90	1116	385	520	179
22	3.08	1510	490	540	175
23	3.32	1125	339	442	133

a. A unit of activity equals a change in OD of 0.001/min.

b. Homogenized in Erway homogenizer.

c. Homogenized with Teflon pestle homogenizer.

d. Centrifuged at 1500 X G for 15 minutes.

e. Spectrophotometer working improperly, values may not be valid.

f. Supernatant from 1500 X G for 30 minutes.

TABLE 2, continued

Sediment 24,500 X G			Supernatant 24,500 X G		
	DPN			TPN	
	Activity[a] Units			Activity[a] Units	
mg N per ml	per ml	per mg N	mg N per ml	per ml	per mg N
0.754	805	1068	1.69	607	359
0.719	880	1224	1.45	530	366
0.753			1.55	447	288
0.79	1096	1307	1.64	640	390
0.85	1140	1341	1.75	616	352
1.04	757	728	2.09	398	190

The volume of the substrate was compensated by water, so that the total volumes in both cells were identical.

In the measurement of the rate of any enzyme activity the activity can be measured either by the disappearance of the reactants or the appearance of the products. Since the coenzymes DPN or TPN are necessary for the oxidation of testosterone by guinea pig liver, it was felt that the rate of formation of DPNH or TPNH would be a good measurement of the rate of conversion of testosterone to 4-androstene-3,17-dione. This method has been used for determining enzymatic transformations of androgens (45, 46). That this method is valid was confirmed (Chapter IV). The rate of formation of DPNH or TPNH is easily followed spectrophotometrically since the reduced form has a molar extinction of 6.22×10^3 at 3400 Å, while the oxidized form has none at this wave length, nor does the testosterone or 4-androstene-3,17-dione.

The thermostatically controlled cell compartment of the Cary was kept at 37°C. and it was demonstrated by actual measurement that the temperature of the reaction mixture was kept at this figure during the time necessary for the study. The reagents were kept in a water bath at 40° C., which was high enough so that the temperature of the reaction mixture dropped to 37°C. during the preparation of the reaction mixture in the cuvettes. The tissue was kept in an ice-bath and the small amount used (0.01 to 0.10 ml.) was not considered great enough to alter the temperature significantly.

The reaction mixture most commonly used, and that set up to be the standard procedure of Chapters V and VI is shown in Table 3. In the experiments in which the steroid was added in ethyl alcohol, 1.75 ml of

TABLE 3

STANDARD REACTION MIXTURE FOR MEASURING THE RATE OF OXIDATION OF TESTOSTERONE

Reaction component	Blank (or reference cell)	Experimental cell
Cofactor (DPN 15 mg/ml) or (TPN 5 mg/ml)	0.20 ml	0.20 ml
Buffer (sodium pyrophosphate 0.18 M, pH 9.5)	1.00 ml	1.00 ml
Substrate (water solution of testosterone assaying 33 μg/ml)	none	1.80 ml
Water	1.80 ml	none
Enzyme (5 to 10 % tissue)	0.01 to 0.10 ml	0.01 to 0.10 ml

water were added to both cells and 0.05 ml of ethyl alcohol was added to the blank, making a total reaction volume of 3.0 ml plus enzyme.

The quantity of tissue preparation added to the reaction mixture was at all times kept low enough that a proportionate increase in rate was obtained from an increased amount of tissue added to the reaction.

Nitrogen determinations were made on the tissue fractions wherever possible. The nitrogen content was determined by the micro Kjeldahl method.

The unit of activity used throughout this dissertation is defined as the change in optical density (OD) at 3400 Å of 0.001 in 1 minute. Activities are expressed in terms of units per ml of guinea pig liver preparation or in terms of units per mg of nitrogen in that preparation.

Preparation of Substrate

The testosterone and other steroids used as substrates were purified on alumina columns followed by crystallization from proper solvents, and their purity was checked by melting point, infra-red spectroscopy and paper chromatography by members of this laboratory. In most of the experiments water solutions of testosterone were used in which the steroid was solubilized by agitation in the Virtis for 30 minutes, allowed to stand for 8-12 hours, then the excess steroid filtered off through washed filter paper Whatman #1. In the first experiments the steroid was stirred for 3 hours, but subsequent studies showed that maximum solubility of testosterone was obtained by agitation for 30 minutes. All subsequent solutions were made employing this length of time for agitation.

Water solutions of 10 other steroids to be used as substrates were prepared. A given known amount (approximately 10 mg) of each of these steroids was stirred in 100 ml of water in the Virtis set at 10 for 3 hours at room temperature. Each solution was then allowed to stand overnight at room temperature, after which it was filtered through washed Whatman #1 filter paper. The concentrations of the steroid in the filtrates were determined by evaporation of given amounts of the water solutions under reduced pressure at 70°C., followed by analysis with either of three methods: (1) extinction at proper wave length, (2) Zimmerman method (47), or (3) chromic acid oxidation followed by Zimmerman. In some cases analyses by two of these methods were compared.

Since the solubility of steroids in water becomes a limiting factor in substrate concentration experiments, some of these studies were carried out using ethyl alcohol solutions of steroids made to the desired concentration by weight.

CHAPTER III

FACTORS WHICH INFLUENCE AND DISTINGUISH THE DPN AND TPN SPECIFIC OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

The methods which may be used to define and characterize enzymes and enzyme systems have been well-developed. An enzyme which is pure according to criteria which are applied to proteins, i.e. solubility and behavior as a single entity in an electrophoretic or centrifugal field, may be distinguished from another enzyme by these criteria. An enzyme which has not been purified cannot be distinguished solely on this chemical and physical behavior, but must be characterized by how changes in the physical-chemical environment affect the enzyme reaction as manifested by changes in rate of the enzyme reaction. Substrate specificity and substrate concentration, cofactor specificity, pH, activators and inhibitors are among the factors which change the physical-chemical environment and will influence the rate of the enzymatic reaction. Thus the rates at which an enzyme will catalyze a reaction under various conditions are characteristic of that enzyme and may be used as criteria to distinguish it from other enzymes which may catalyze similar reactions.

A number of factors are considered in this chapter which influence the rate of enzyme reaction by changing the physical-chemical

environment and thus help characterize and distinguish between two similar reactions or activities which have been observed to be catalyzed by guinea pig liver. These are specifically the use of either DPN or TPN as coenzyme in the enzymatic reversible conversion of testosterone to 4-androstene-3,17-dione by guinea pig liver or fractions thereof.

In measuring the effect of a reaction component on the rate of the reaction it is more convenient, for simpler measurements and calculations, to make the reaction pseudo first order with respect to this component. Thus, if the rate is to be studied the reaction mixtures are so designed as to make the concentration of enzyme the rate limiting factor, with all the other components being in excess. The time of the measurement is restricted to a short enough time so that the concentrations of the reaction components do not change significantly during this period.

The experiments of this chapter were designed to determine the concentration of reactants necessary to satisfy these qualifications and at the same time to compare and distinguish between the two reactivities described above.

Buffer Concentration

Testosterone Used for Substrate

Procedure. The effect of buffer concentration on the rate of oxidation of testosterone was studied by the measurement of either the DPNH or TPNH formed from DPN or TPN respectively. The rate of oxidation using liver homogenate as the enzyme source was studied with both

coenzymes, DPN and TPN, while the rate of oxidation using the mitochondrial fraction from guinea pig liver as the enzyme source was studied with DPN and the supernatant 24,500 X G fraction was studied with TPN as coenzyme. The methods employed in the measurement of the rates of these reactions were in general those described in Chapter II.

The concentration of the coenzyme used was 300 μg/3 ml reaction mixture. Thirty μg of testosterone in aqueous solution were used as the substrate in the 3 ml reaction mixture. The buffer employed in these reactions was sodium pyrophosphate buffer, pH 9.0. This was the measured pH of the buffer before the addition of reaction components. Later experiments showed that this was not necessarily the true pH of the reaction mixture (Table 6). Graduations in concentration of buffer varied from 0.015 to 0.21 mMoles per 3 ml reaction mixture.

Results. The results of the studies made to determine the effect of buffer concentration on the rate of oxidation of testosterone are tabulated in Table 4 and illustrated by Figures 4 and 5. The activities of both the mitochondrial and the homogenate fractions using DPN cofactor appear to be affected in the same fashion and to the same degree by varying the buffer concentration. The rate of activity is seen to rise with increasing concentration of buffer until a level of activity is reached which is maximum from a concentration of 0.09 mM to 0.21 mM per reaction. This increase in activity amounts to roughly 90 per cent.

The activities of the supernatant and homogenate fractions using TPN as cofactor also appear to be affected in the same manner by the buffer concentrations. These again rise until a buffer concentration of

TABLE 4

THE EFFECT OF BUFFER CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Buffer Concentration	Units Activity per mg N			
	Homogenate		Mitochondria	Supernatant
mMoles per 3 ml	DPN*	TPN*	DPN*	TPN*
0.015	105	84	247	140
0.030	140	99	388	163
0.060	164	113	409	150
0.09	185	123	455	136
0.12	184	116	494	180
0.15	180	103	444	165
0.18	195	129	452	167
0.21	191	120	447	145

*DPN and TPN concentrations were 300 μg/reaction (3 ml)

0.09 mMoles is reached, with no further increment in activity as buffer concentration is increased. However, the magnitude of the change is not as great in the TPN system. There is approximately a 30 per cent change.

Since there appears to be a wide range of buffer concentrations which may be used to give the maximum enzyme activity, a concentration intermediate in that range was selected to be used in the experiments which follow.

Later experiments (Table 6) would lead one to believe that the

FIGURE 4
THE EFFECT OF BUFFER CONCENTRATION ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE

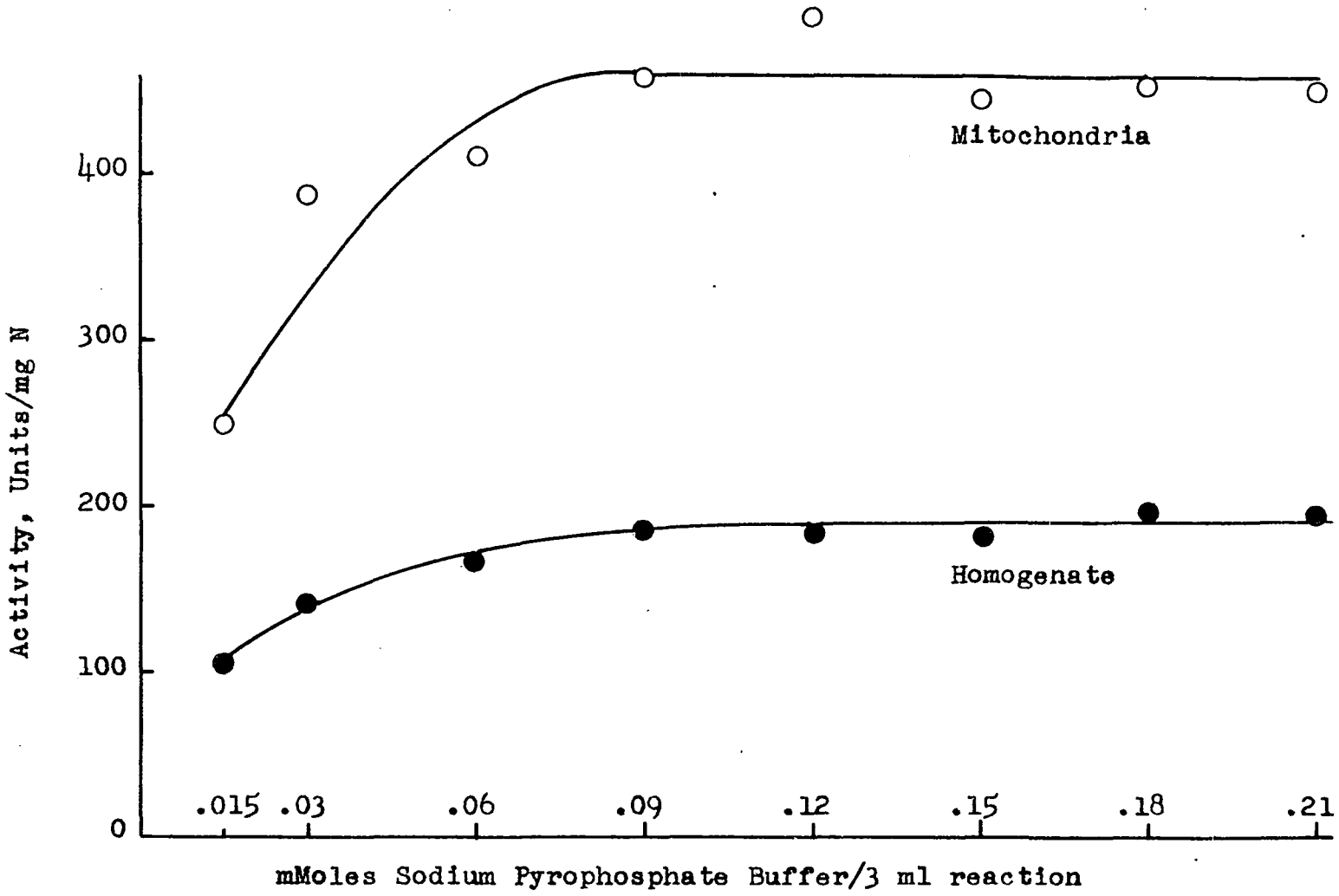
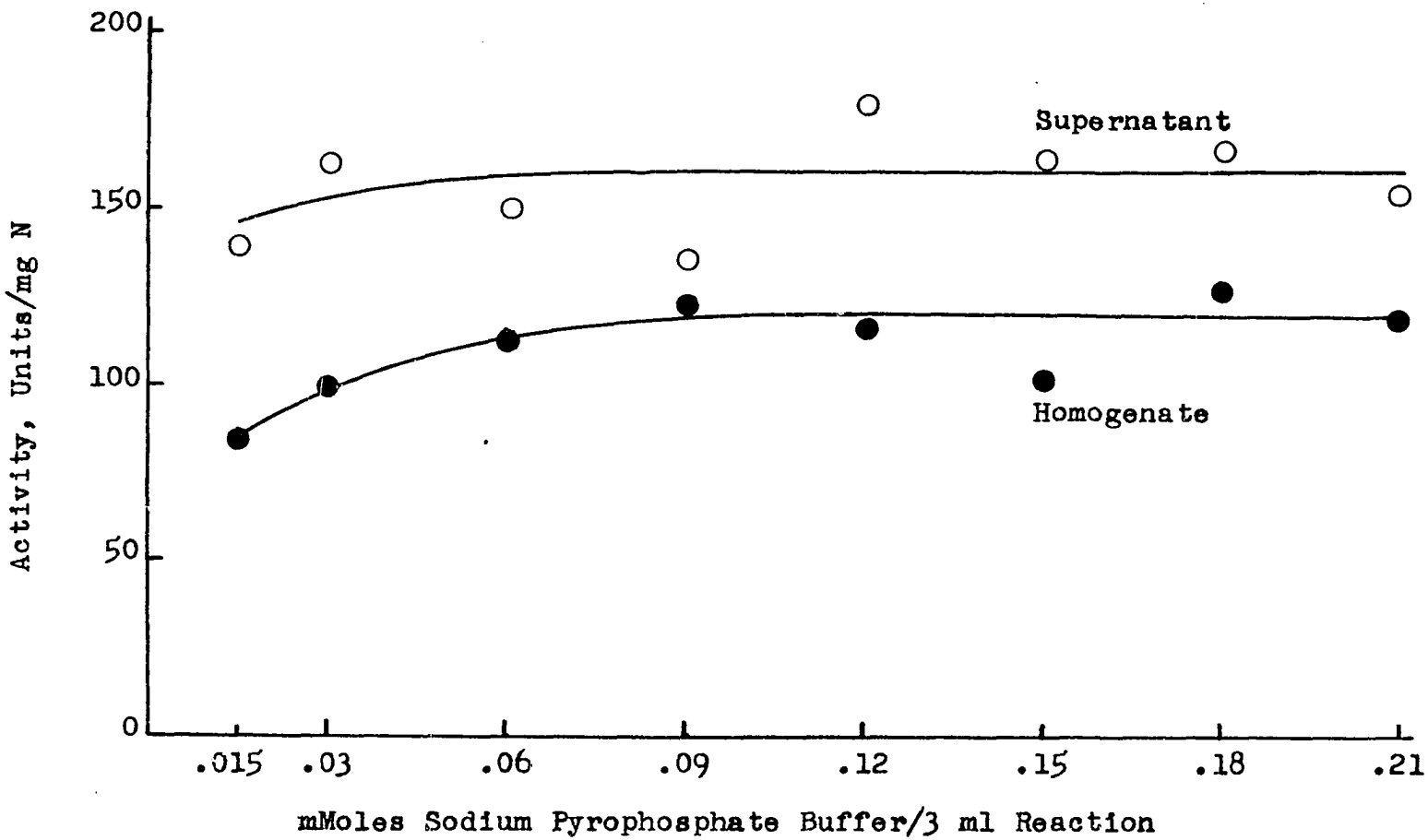


FIGURE 5

THE EFFECT OF BUFFER CONCENTRATION ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE



conditions attributed here to optimum concentration may in reality be associated with the better buffering capacity of the high concentration of buffer.

4-Androstene-3,17-dione Used for Substrate

Procedure. The effect of buffer concentration on the rate of reduction of 4-androstene-3,17-dione was studied via the measurement of the disappearance of TPNH as followed spectrophotometrically at 3400 Å, utilizing 4-androstene-3,17-dione as the substrate. The source of the enzyme used was the guinea pig liver supernatant 24,500 X G. Reduced TPN was made by the modified method of Green and Dewan (49) and analyzed 80 per cent reduced TPN. The concentration of the cofactor used was 300 µg/3 ml reaction mixture. Thirty µg of 4-androstenedione in aqueous solution were used as the substrate in the reaction mixture. The buffer employed was sodium phosphate buffer pH 6.5, varying in concentration from 0.015 to 0.35 mMoles per 3 ml reaction mixture. The pH 6.5 refers to the pH of the buffer before the remaining reaction components were added and it was discovered in later experiments not to be the true pH of the reaction mixture.

Results. The results of this study are given in Table 5 and illustrated by Figure 6. The rate of the reaction is increased approximately 40 per cent with increased buffer concentration. The optimum buffer concentration is also exhibited over a wide concentration range.

Hydrogen Ion Concentration

Testosterone Used for Substrate

Procedure. The effect of hydrogen ion concentration (pH) on the

TABLE 5

THE EFFECT OF BUFFER CONCENTRATION ON THE RATE OF REDUCTION OF 4-ANDROSTENE-3,17-DIONE BY GUINEA PIG LIVER SUPERNATANT

Buffer[a] Concentration mMoles/3 ml reaction	Activity[b] Units[c]/mg N
0.015	10.7
0.03	12.0
0.06	14.2
0.12	14.5
0.18	15.1
0.24	15.5
0.30	15.1
0.35	16.7

a. Sodium phosphate buffer pH 6.5

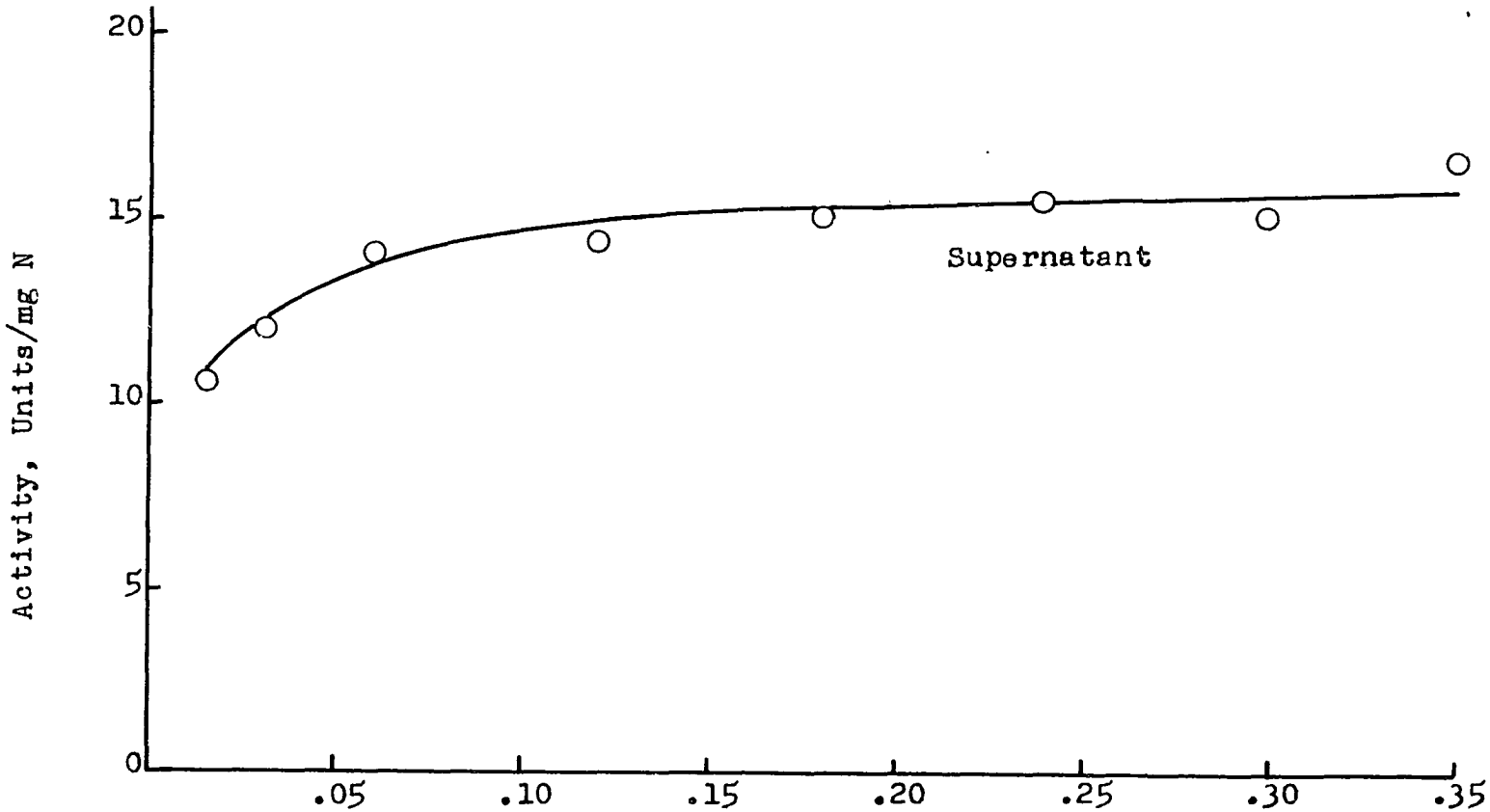
b. Cofactor TPNH, 300 μg/3 ml reaction, 80 per cent reduced.

c. The unit of activity equals a change in OD of 0.001/min.

rate of oxidation of testosterone by guinea pig liver fractions was studied utilizing testosterone as the substrate via the measurement of either the DPNH or the TPNH formed from DPN or TPN respectively. The buffer employed in these reactions was sodium pyrophosphate buffer, 0.18 mMoles per 3 ml reaction mixture, varying in pH from 8.0 to 11.5. This initial pH of the buffer was shown not to represent the true pH of the

FIGURE 6

THE EFFECT OF BUFFER CONCENTRATION ON THE RATE OF THE TPNH-LINKED REDUCTION OF 4-ANDROSTENE-3,17-DIONE



reaction medium, and therefore, the actual pH of the reaction mixture was measured and the data plotted from that pH. The pH was measured with either a Beckman Model H or Zeromatic pH meter, both equipped with small electrodes for 3 ml volumes.

Several concentrations of cofactor (DPN or TPN) were used in the pH experiments. The concentration of 300 μg DPN or TPN/3 ml was used in the first experiments. Later experiments (Figures 14, 15 and 16) demonstrated that this was a limiting cofactor concentration and, therefore, the experiments were repeated using 3000 μg DPN per 3 ml reaction volume and 1000 μg TPN per 3 ml reaction mixture. Due to the lessened ability to accurately control the pH of the DPN mixture when 3000 μg were used, the experiment was repeated using 1500 μg DPN/3 ml reaction.

Results. The effect of the hydrogen ion concentration on the oxidation of testosterone by guinea pig liver preparations using DPN as the coenzyme has been given in Tables 6, 7, and 8 and illustrated by Figures 7, 8, 9, and 10. The effect of pH, using TPN as the coenzyme has been given in Tables 9 and 10 which are illustrated by Figures 11 and 12. The data have been given both for the homogenate and the mitochondrial fractions using DPN and for the homogenate and supernatant (24,500 X G) fractions using TPN on several guinea pig liver preparations which are listed in Chapter II. The data are expressed in terms of the mg of N found in the fractions. Whenever possible the actual pH of the reaction mixture is given; otherwise the initial pH of the buffer is given in parenthesis.

TABLE 6

THE EFFECT OF pH ON THE RATE OF THE DPN[a]-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Prep 5		Prep 6			Prep 7	
pH	Activity Units per mg N	pH		Activity Units per mg N	pH	Activity Units per mg N
			Homogenate			
8.33	73	8.36		94	8.35	154
8.41	73	8.83		225	8.85	224
8.82	122	9.31		392	8.88	254
9.27	254	9.60		409	9.38	456
9.59	276	9.79		34	9.45	518
9.79	8	9.98		17	9.60	478
					9.80	17
			Mitochondria			
(8.0)	194	(8.0)	8.34[b]	266		
(8.5)	256	(8.5)	8.82[b]	417		
		(9.0)	9.30[b]	504		
9.33	527	(9.5)	9.55[b]	411		
9.63	583	(10.0)	9.73[b]	150		
9.84	122	(10.3)	9.9[b]	75		
9.91	36					

a. DPN concentration 300 μg / 3 ml reaction

b. Average pH of homogenate reactions

() pH of buffer before addition to reaction mixture, others are actual pH of reaction mixture.

TABLE 7

THE EFFECT OF pH ON THE RATE OF THE DPN[a]-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Prep. 12

Homogenate		Mitochondria	
pH	Activity Units per mg N	pH	Activity Units per mg N
8.64	48	8.58	219
8.66	51	8.7	261
9.0	80	9.0	360
8.95	84	9.0	386
9.19	137	9.25	610
9.22	126	9.28	447
9.35	212	9.38	629
9.33	200	9.39	666
9.40	231	9.45	686
9.42	225	9.45	708
9.49 (10.5)	262	9.5	807
(9.45)	251	9.5	784
9.91	Denatured after 3 minutes	10.0	114 (Den)
10.04	no activity	10.0	Denatured
		10.5	Denatured

a. DPN concentration 3000 µg / 3 ml reaction

() pH of 0.18 M sodium pyrophosphate buffer before addition to reaction mixture, all others being measured pH of the reaction mixtures.

TABLE 8

THE EFFECT OF pH ON THE RATE OF THE DPN[a]-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Prep. 14				Prep. 13	
Homogenate		Mitochondria		Homogenate	
pH	Activity[b] Units per mg N	pH	Activity[b] Units per mg N	pH	Activity[b] Units per mg N
7.8	15	7.8	119	7.9	182
8.3	62	8.3	235	8.35	219
8.7	99	8.71	386	8.8	260
9.15	193	9.15	699	9.3	323
9.42	319	9.43	949	9.4	366
9.5	354	9.5	941	9.5	411
(9.6)	394	9.6	1053	9.65	435
9.7	365[c]	9.85	Denatured	9.7	416
10.05	Denatured	10.1	Denatured	9.75	331[c]
				9.75	424
				9.85	454
				10.1	12

a. DPN concentration 1500 μg/3 ml reaction

b. A unit of activity equals a change in OD of 0.001 per min.

c. Reaction was non-linear

() pH of 0.18 M sodium pyrophosphate buffer before addition to reaction mixture

FIGURE 7

THE EFFECT OF pH ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE BY HOMOGENATE USING SUB-OPTIMUM COFACTOR CONCENTRATION

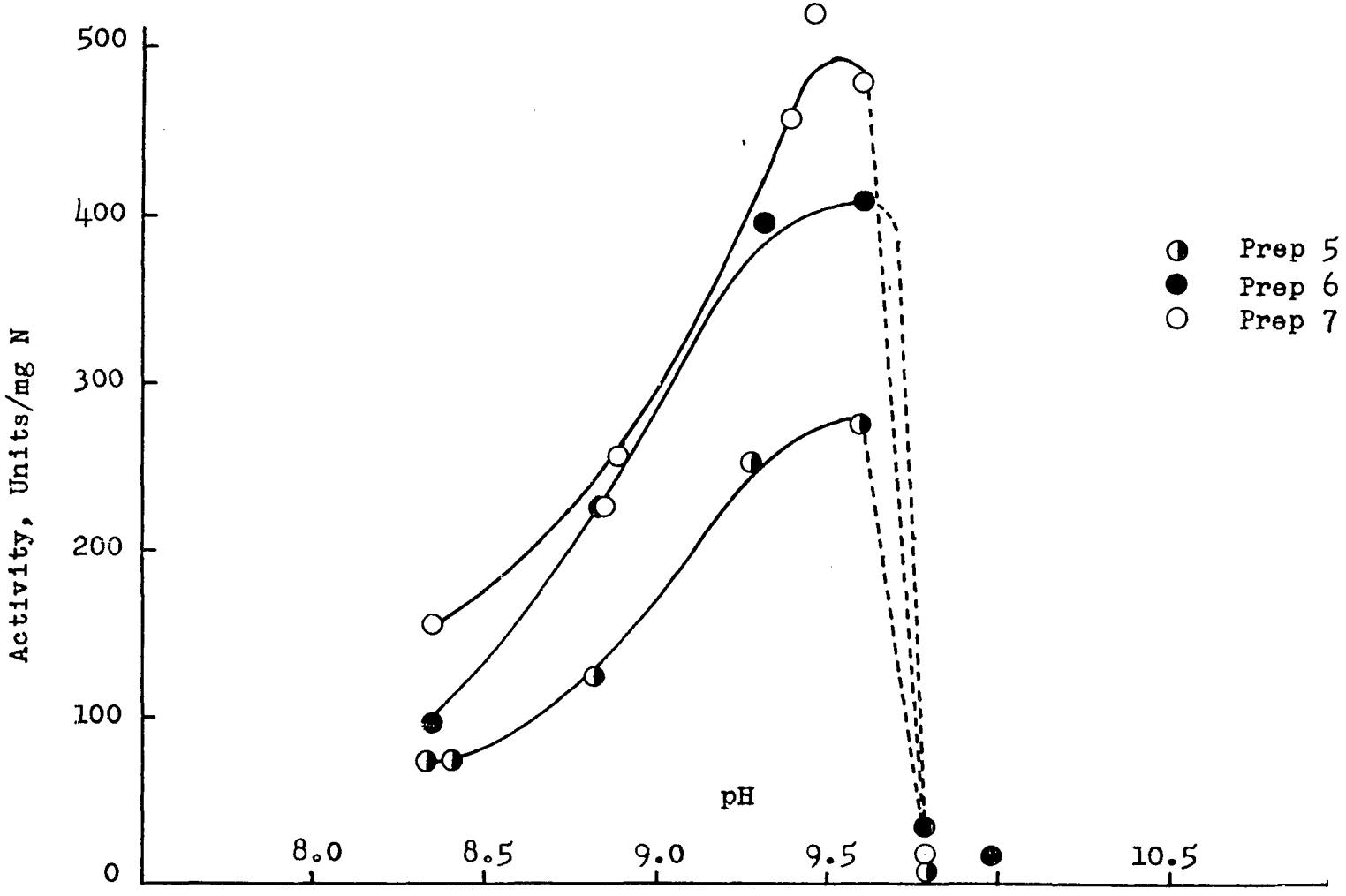


FIGURE 8
THE EFFECT OF pH ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE BY MITOCHONDRIA WITH SUB-OPTIMUM COFACTOR CONCENTRATION

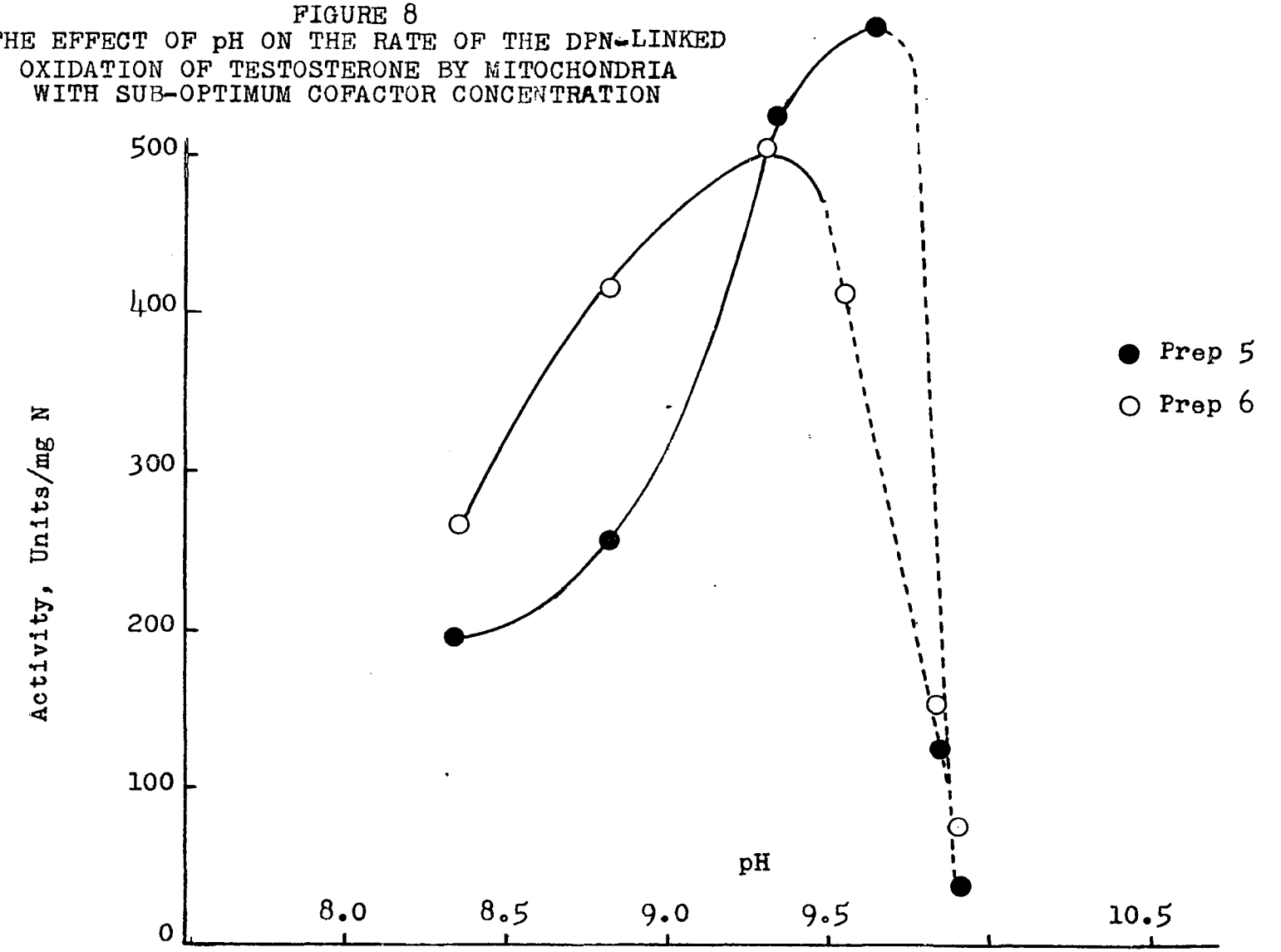


FIGURE 9
THE EFFECT OF pH ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE BY HOMOGENATE WITH OPTIMUM COFACTOR CONCENTRATION

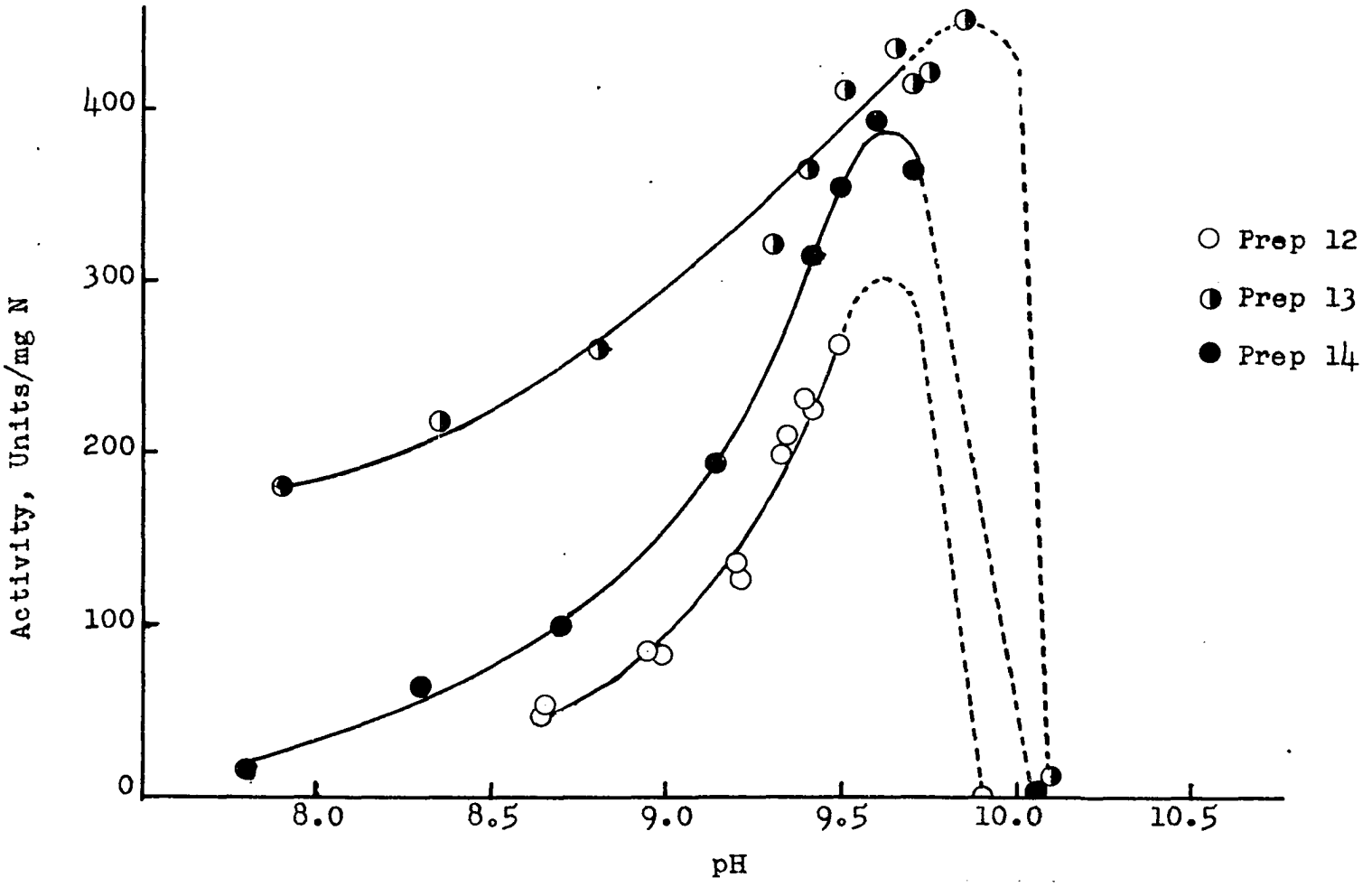


FIGURE 10

THE EFFECT OF pH ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE BY MITOCHONDRIA WITH OPTIMUM COFACTOR CONCENTRATION

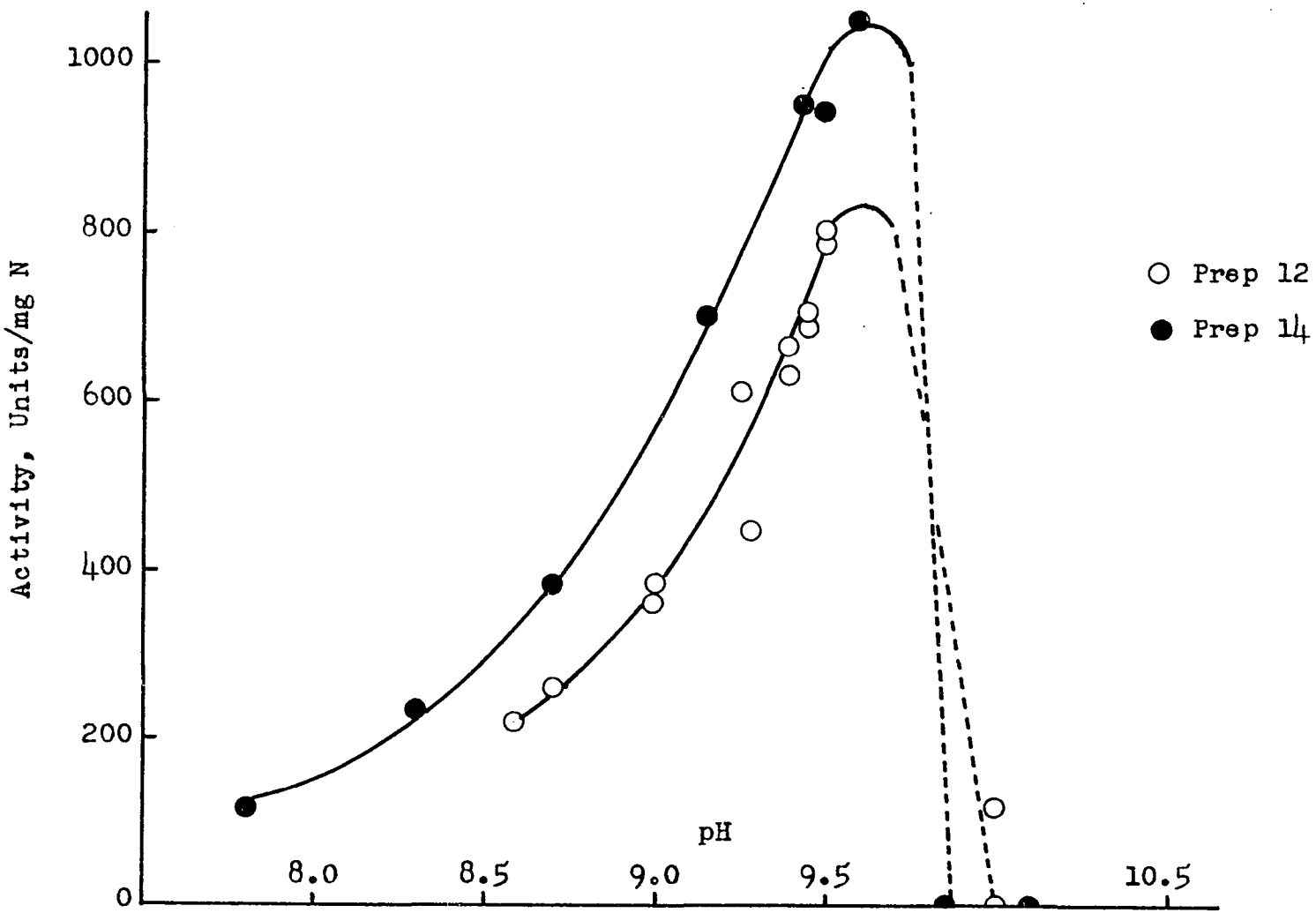


TABLE 9

THE EFFECT OF pH ON THE RATE OF THE TPN[a]-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Homogenate				Supernatant			
Prep. 5		Prep. 6		Prep. 5		Prep. 6	
pH	Activity[b] Units per mg N	pH	Activity[b] Units per mg N	pH	Activity[b] Units per mg N	pH	Activity[b] Units per mg N
8.31	80	8.30	47	(8.0)	49	8.32	157
8.79	115	8.75	96	(8.5)	79	8.80	263
9.25	129	9.30	162	(9.0)	127	9.28	392
9.51	153	9.50	174	9.5	144	9.52	327
9.70	119	9.69	196	(10.0)	153	9.58	360
9.88	123	9.80	185			9.72	390
						9.90	370

a. TPN concentration 300 µg/3 ml reaction

b. A unit of activity equals the change in OD of 0.001/min.

() pH of 0.18 M sodium pyrophosphate buffer before addition to reaction mixture

TABLE 10

THE EFFECT OF pH ON THE RATE OF THE TPN[a]-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Prep. 12			
Homogenate		Supernatant	
pH	Activity[b]	pH	Activity[b]
8.28	72	8.40	123
8.35	74	8.35	142
8.78	110	8.75	203
8.79	109	8.82	193
9.04	137	9.15	266
9.07	141	9.18	279
9.23	137	9.50	321
9.26	143	9.50	302
9.30	154	9.7	350
9.33	173	9.72	331
9.38	148	9.75	346
9.41	146	9.95	319
9.68 (11.1)	166	9.92	337
(11.1)	165	10.0	310
(11.5)	181	10.1	318
9.9 (11.55)	194	11.55	11
10.1	183		
11.5	0		

a. TPN concentration 1000 µg / 3 ml reaction

b. Activity expressed as Units / mg N.

() pH of 0.18 M sodium pyrophosphate buffer before addition to reaction mixture

FIGURE 11

THE EFFECT OF pH ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE BY HOMOGENATE

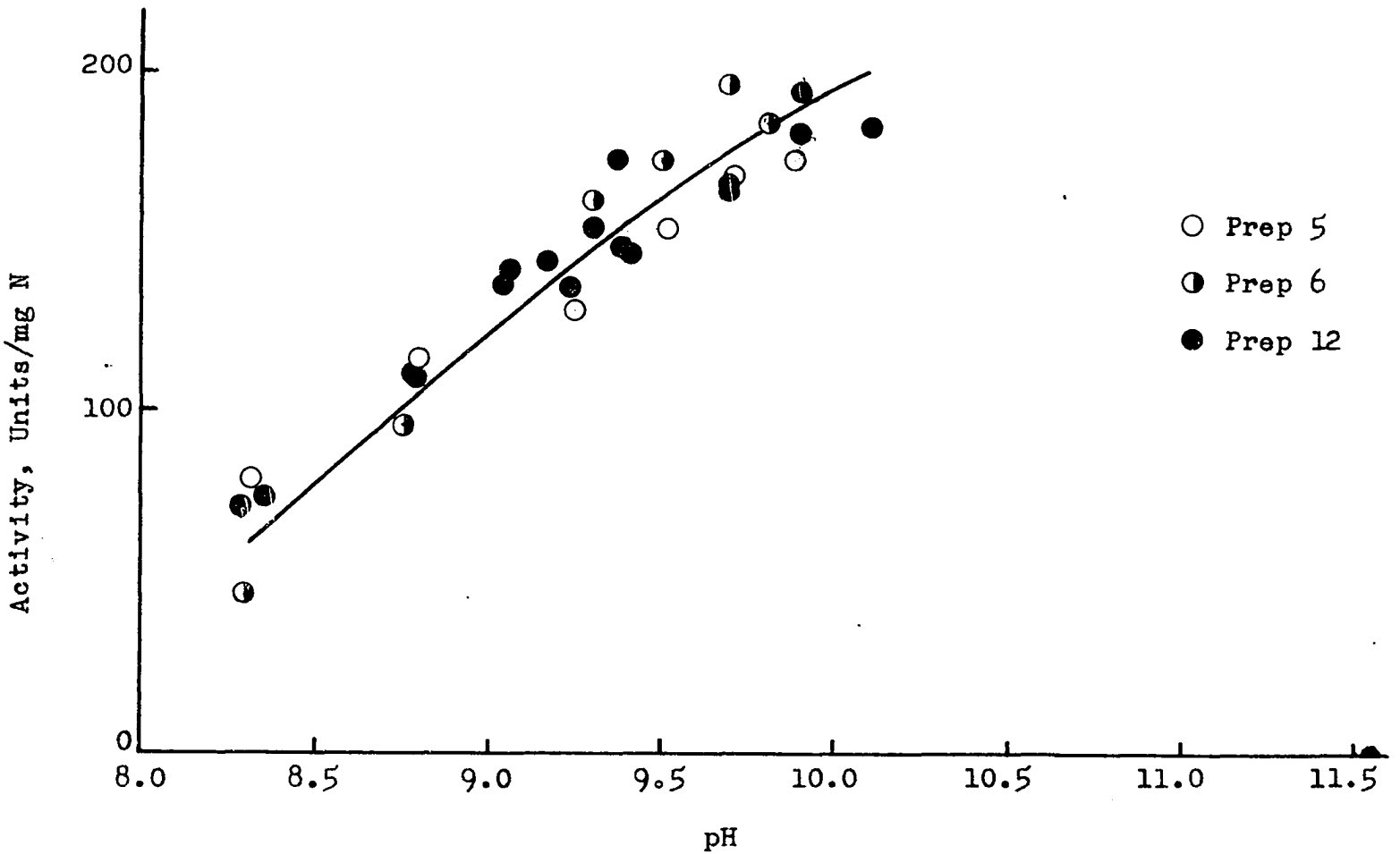
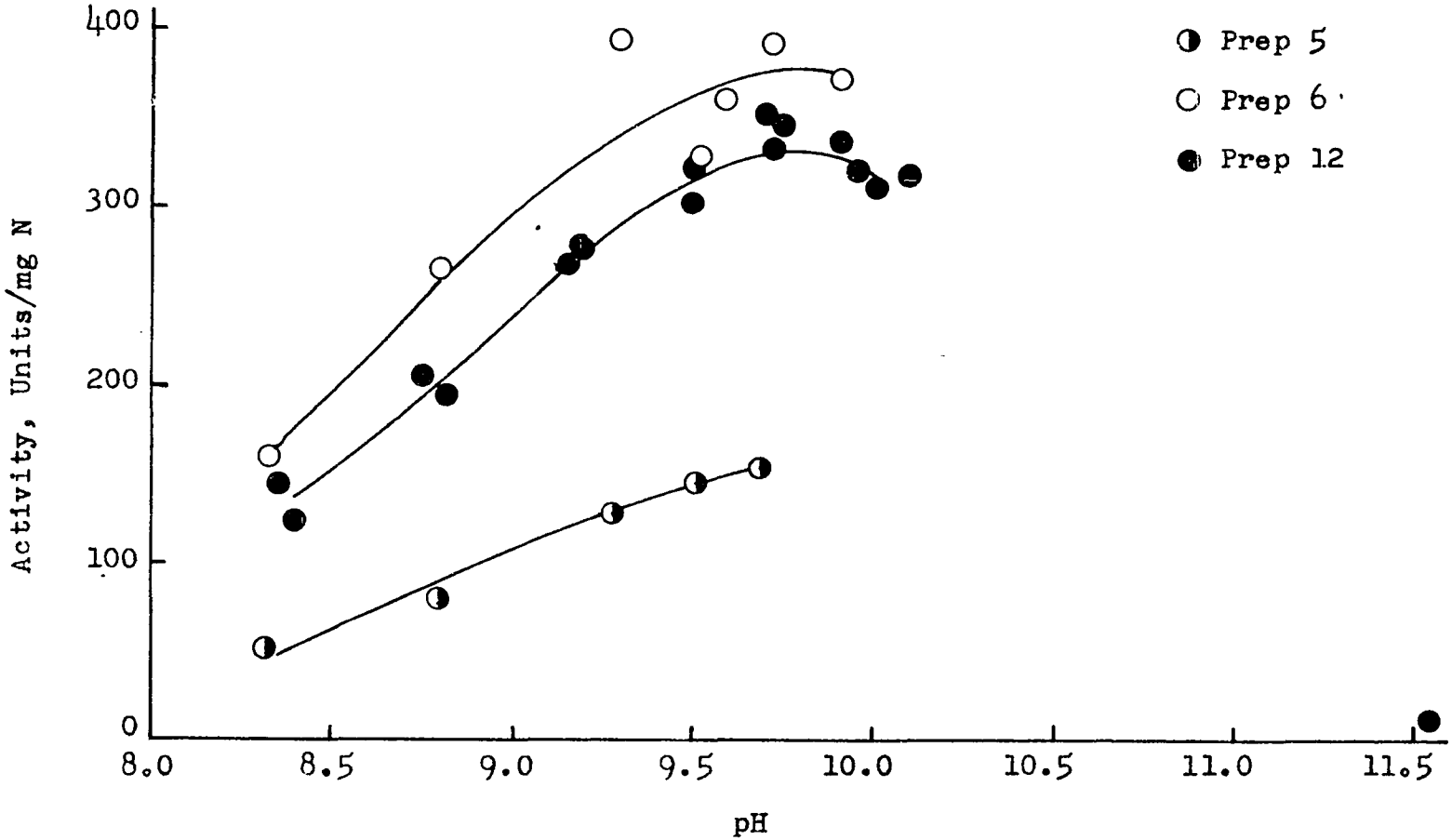


FIGURE 12
THE EFFECT OF pH ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE BY SUPERNATANT



The DPN linked enzyme activity is shown to be very markedly affected by the pH of the reaction mixture, both in the homogenate and the mitochondrial fractions. It was shown to rise progressively with the increasing pH until a point is reached at which denaturation of the tissue takes place. The pH at which the maximum activity occurred before denaturation of the tissue appeared to be about pH 9.6 with the two lower concentrations (300 and 1500 μg/reaction) of DPN used. However, with the highest concentration of DPN (300 μg) it was very difficult to control the pH above 9.5 and there is a large gap in the data in the region from pH 9.5 to 10.0. At pH 9.6 there is an approximately 200-300 per cent increase in activity over pH 8.5.

The mitochondria exhibits a pH dependency similar to the homogenate with all three concentrations of DPN used.

The TPN linked enzyme activity is shown to be influenced by pH but not in the same way nor to the same extent as the DPN linked enzyme activity. The rise in activity with increasing pH is not as sharp in the TPN system as in the DPN system and there is a broad region of maximum activity between pH 9.5 and 10.0. At pH 9.6 there is an approximately 100 per cent increase over the activity at pH 8.5 in the TPN system. It is to be noted that there is activity at pH 10.0 in the TPN system whereas there is no activity above pH 9.8 in the DPN system. Increasing the pH above 10.0 did, however, result in denaturation of the tissue.

The supernatant exhibits a pH dependency similar to the homogenate with the TPN system.

4-Androstene-3,17-dione Used for Substrate

Procedure. The effect of pH on the rate of the reverse reaction was studied by measuring the rate of disappearance of DPNH or TPNH and utilizing 4-androstene-3,17-dione as the substrate. The buffer employed in these reactions was 0.12 mMoles of sodium phosphate buffer per 3 ml reaction mixture, varying in pH from 3.0 to 7.5. Again the true pH of the reaction mixture is not represented by the pH of the initial buffer added and the measured pH is given.

The mitochondrial fraction was studied using a cofactor concentration of 300 μg per 3 ml reaction of DPNH which was 94 per cent reduced. The supernatant fraction was studied using 300 μg of TPNH which was 83 per cent reduced.

Results. The effect of pH on the rate of reduction of androstenedione by the mitochondrial and supernatant fractions utilizing DPNH and TPNH respectively is given in Table 11 and illustrated in Figure 13. There is a sharp rise in enzyme activity both with the DPNH and the TPNH system as the pH is lowered from 7.5. Below pH 5.2 there was a tendency for denaturation to take place with the TPNH system while the DPNH system maintained activity to pH 3.3. There is an approximate 10 fold increase with the DPNH system from pH 7.5 to 3.3, while the maximum activity attained with both systems is approximately equal.

Cofactor Concentration

Procedure

The effect of coenzyme (DPN or TPN) concentration on the rate of

TABLE 11

THE EFFECT OF pH ON THE RATE OF THE DPNH- AND TPNH-LINKED REDUCTION OF 4-ANDROSTENE-3,17-DIONE BY GUINEA PIG LIVER

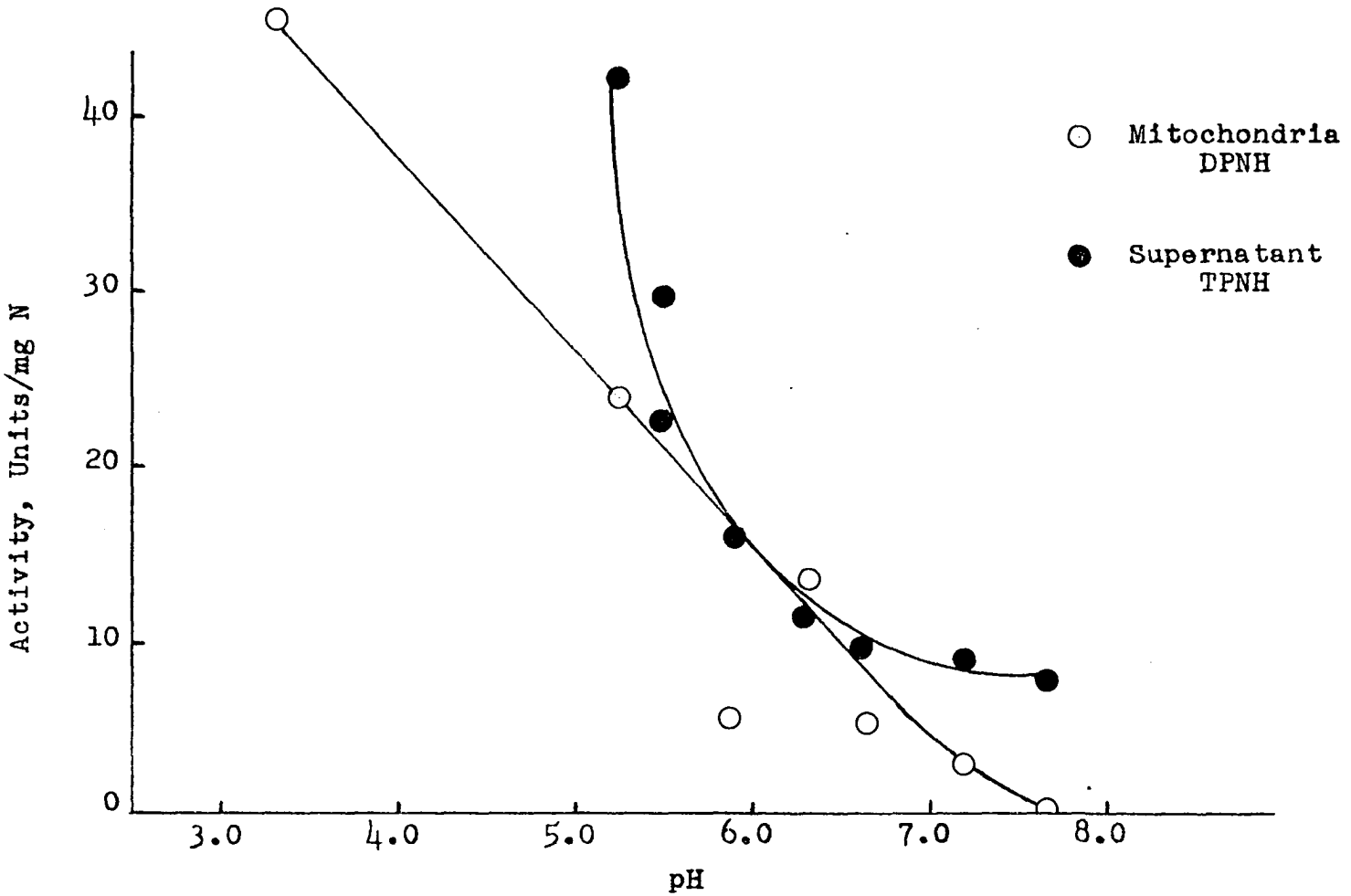
Prep. 5			
Supernatant		Mitochondria	
300 µg TPNH (83% reduced)		300 µg DPNH (94% reduced)	
pH	Activity[a]	pH	Activity[a]
5.23	42	3.30	46
5.5[b]	30, 26	5.25	24
5.9	16	5.87	6
6.3	11	5.88	6
6.62	10	6.33	14
		6.65	5
7.20[b]	9	7.20	3
7.67[b]	8	7.67	0

a. Activity expressed as Units/mg N. A unit of activity equals a change in OD of 0.001/minute.

b. pH taken from mitochondria data

oxidation of testosterone by guinea pig liver was studied utilizing testosterone as the substrate by the measurement of either the DPNH or the TPNH formed from DPN or TPN respectively. The guinea pig liver homogenate was studied with both cofactors, DPN and TPN, while the mitochondrial fraction was studied with DPN and the supernatant fraction was studied with TPN as cofactor. A tissue concentration in the

FIGURE 13
THE EFFECT OF pH ON THE RATE OF THE DPNH- AND TPNH-LINKED REDUCTION OF 4-ANDROSTENE-3,17-DIONE



region of 0.02 to 0.04 ml of 10 per cent (weight-volume) preparation per reaction mixture was used for the determinations.

The buffer employed in these reactions was 0.18 M sodium pyrophosphate buffer at a pH sufficient to maintain the reaction at 9.50. It was found, however, that at higher concentrations of cofactor the pH of the reaction mixture was lowered and, therefore, the data for preps 8 and 9 were corrected for pH differences, and buffer with several different pH levels was used with prep 17 in order to maintain a constant pH of 9.50 in the reaction vessel.

A volume of 0.9 ml of a water solution of testosterone assaying 34.4 μg/ml was used with prep 8. The concentration was changed to 1.8 ml of testosterone solution assaying 33 μg/ml for preps 9 and 17.

The concentrations of cofactor studied varied from 150 to 4500 μg/reaction for both DPN and TPN.

Results

The effect of cofactor concentration on the rate of oxidation of testosterone by guinea pig liver preparations using DPN as the cofactor has been given in Table 12 and illustrated by Figures 14 and 15 and using TPN as the cofactor has been given in Table 13 and illustrated by Figure 16. The guinea pig liver preparations used are listed in Chapter II, Table 2. The data are expressed in terms of activity per mg N found in the fraction and have been corrected when necessary for the pH effect noted in that section of this chapter.

The DPN-linked activity in the mitochondria and homogenate shows a clear-cut dependency on the concentration of DPN in the reaction.

TABLE 12

THE EFFECT OF COENZYME (DPN) CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

DPN	Activity*				
	Homogenate			Mitochondria	
µg/3 ml reaction	Prep. 8	Prep. 9	Prep. 17	Prep. 8	Prep. 9
150	102		116	390	160
300	200	150	236	585	340
450			274		
600	279	229	323	729	663
750			346		
900	302	237	368	838	795
1050			384		
1200	306		386	895	
1500		290	394		845
1800			416		
2100			408		
2250		263			946
2400	295		384	918	
2700			410		
3000		267	414		1008
3300	314			898	
3750		264			
4500					1013

*Activities are expressed as Units/mg N and corrected for pH changes when necessary. A unit of activity equals a change in OD of 0.001 per minute.

FIGURE 14

THE EFFECT OF COENZYME (DPN) CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE BY HOMOGENATE

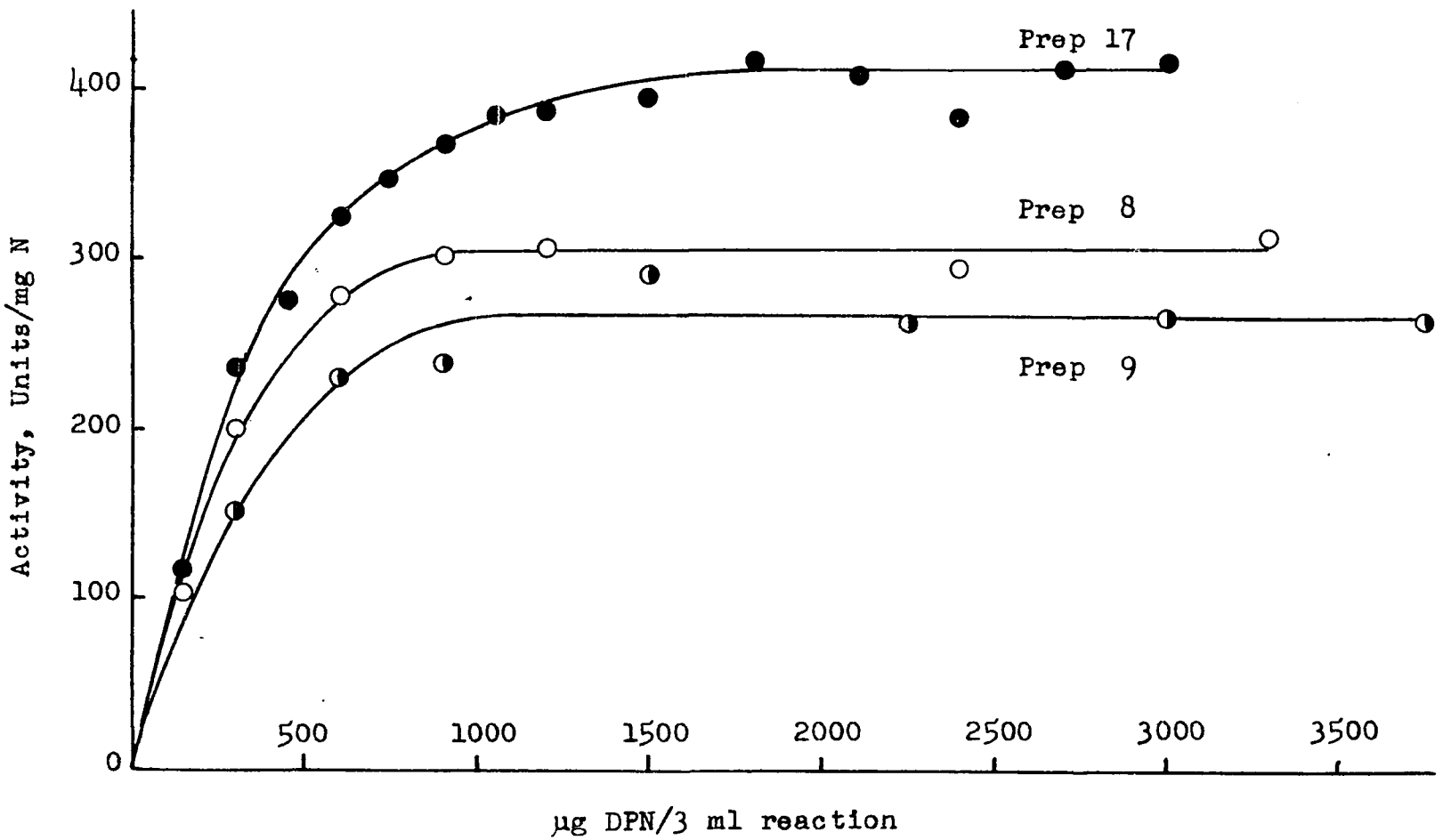


FIGURE 15
THE EFFECT OF COENZYME CONCENTRATION (DPN) ON THE RATE OF OXIDATION OF TESTOSTERONE BY MITOCHONDRIA

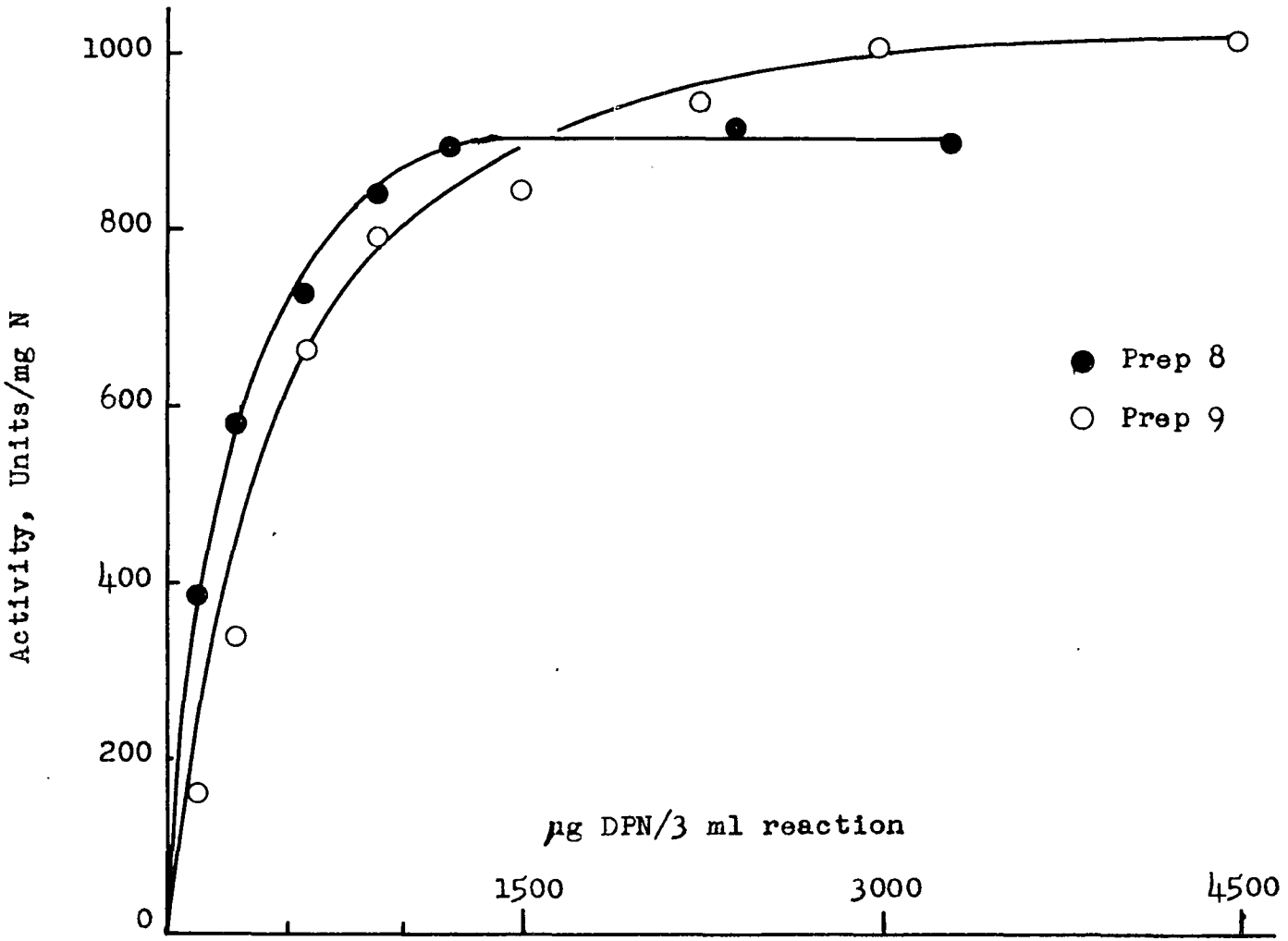


TABLE 13

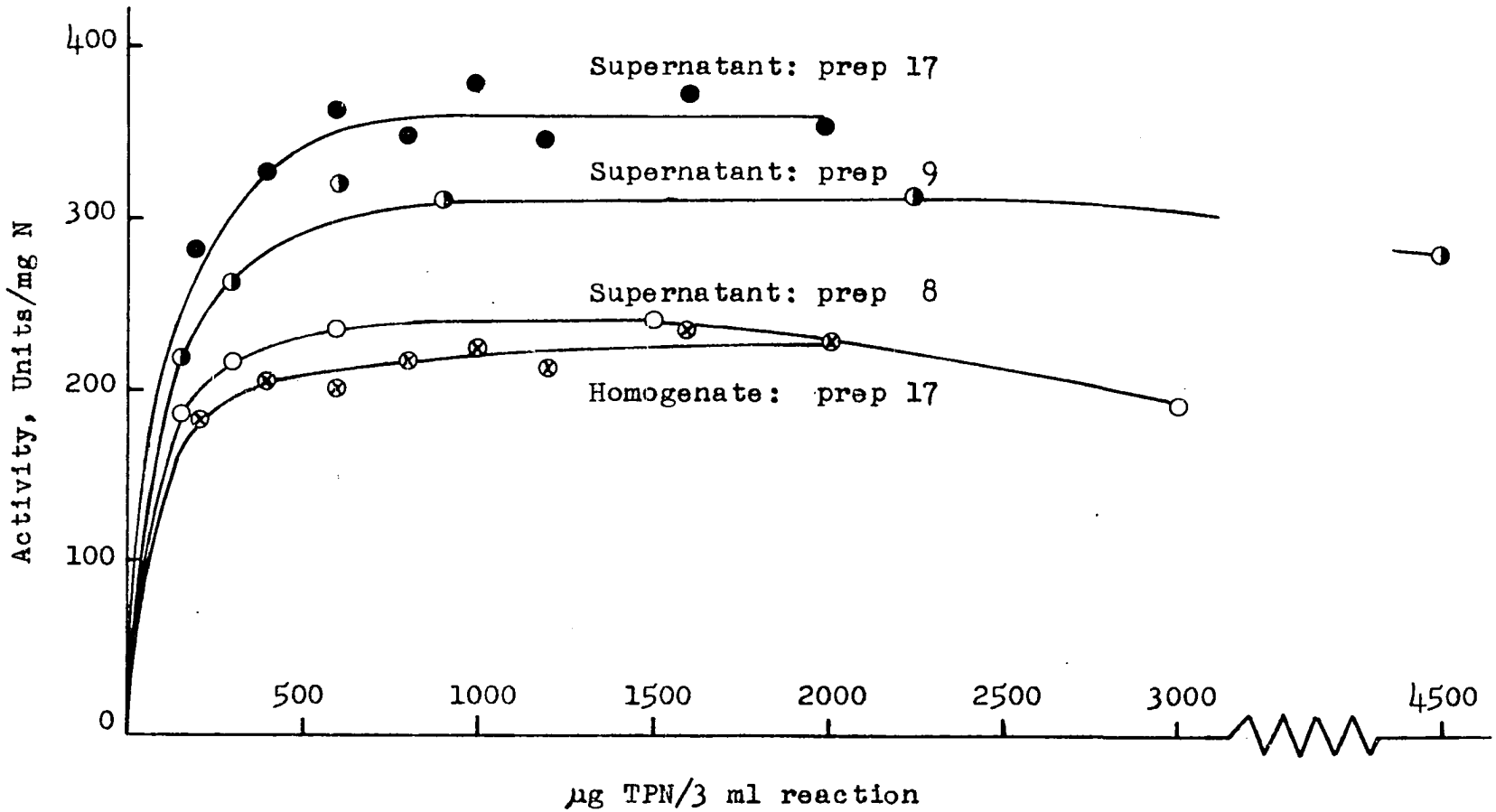
THE EFFECT OF COENZYME (TPN) CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

TPN	Activity* Units/mg N			
µg/3 ml	Homogenate	Supernatant		
reaction	Prep. 17	Prep. 8	Prep. 9	Prep. 17
0				0
150		187	219	
200	182			284
300		216	262	
400	206			327
600	200	236	320	363
800	218			347
900			310	
1000	224			379
1200	213			345
1500		240		
1600	234			371
2000	229			353
2250			309	
3000		190		
4500			276	

*A unit of activity equals a change in OD of 0.001 per minute.

FIGURE 16

THE EFFECT OF COENZYME (TPN) CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE



The activity of both homogenate and mitochondria increases sharply with increasing concentration of DPN until a concentration of approximately 1000 μg/3 ml reaction mixture is reached, at which concentration the maximum activity is reached and the DPN concentration is no longer a limiting factor (Figures 14 and 15). Further increasing the concentration of DPN did not affect the activity in any way except through pH effects.

The TPN-linked enzyme activity in the supernatant and the homogenate also exhibits the same dependency on cofactor concentration for maximum activity, although it would appear that the concentration necessary for maximum activity is of the order of half of that necessary in the DPN system (Figure 16). Both supernatant and homogenate reach maximum activity at about 500 μg TPN/3 ml reaction.

From these studies the concentrations of 3000 μg of DPN and 1000 μg of TPN per 3 ml reaction were chosen as concentrations to be used as standard procedure in the following experiments.

Nicotinamide

Procedure

Since the DPN and TPN linked enzymes were directly dependent on the concentration of cofactor in the reaction mixture, experiments were done to determine whether or not the cofactor could be "protected" by the use of nicotinamide in the reaction mixture. The reaction was run using two levels of cofactor concentration, one a high, non-rate-limiting concentration of cofactor and, another, a low, limiting concentration of cofactor. The concentration of nicotinamide was varied

from 0 to 6 x 10^{-3} mM/3 ml reaction. The levels of cofactor which were used were 300 and 1500 µg DPN per 3 ml reaction mixture and 100 and 1000 µg TPN/3 ml reaction. The pH was kept constant at 9.5 with 0.18 M. pyrophosphate buffer. A water solution (1.8 ml) of testosterone assaying 33.2 µg/ml was used in both systems.

Results

The effect of nicotinamide on the DPN linked rate of oxidation of testosterone by guinea pig liver homogenate and mitochondria is given in Table 14, Figure 17. Nicotinamide would seem to enhance the activity of the homogenate, at the most, approximately 10 per cent, while it in no way alters the activity in the mitochondria. Should the nicotinamide be protecting the DPN from nucleotidase destruction, then supposedly the experiment using the low concentration (300 µg) of DPN would show the sharpest effects. However, this was not so, the effects being about the same in both instances.

The effects of nicotinamide on the TPN linked rate of oxidation of testosterone by guinea pig liver fractions is given in Table 15 and Figure 18. There would seem to be a slight decrease in activity due to the addition of nicotinamide. The decrease would amount to less than 10 per cent of the total activity. Further increasing the amount of nicotinamide added, however, had no further effect.

The effects of nicotinamide were not of such magnitude to warrant using it in the reaction mixture as a standard procedure.

TABLE 14

THE EFFECT OF NICOTINAMIDE ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Nicotinamide μ Moles per reaction	Activity* in Units/mg N			
	Homogenate		Mitochondria	
	DPN (300 μg per reaction)	DPN (1500 μg per reaction)	DPN (300 μg per reaction)	DPN (1500 μg per reaction)
0	183	301	302	809
2.1	179	283	292	861
4.5	183	337	365	861
9.0	205	350	323	829
13.5	202	355	339	871
18.0	201	353	308	850

*A unit of activity equals the change in OD of 0.001 per minute.

Substrate Concentration

Water Solution of Testosterone

Procedure. The effect of substrate concentration on the rate of oxidation of the substrate by guinea pig liver fractions was determined using a water solution of testosterone assaying 32.8 μg/ml. Varying amounts of this solution were used to give a concentration range from 3 to 60 μg/3 ml reaction. The homogenate and mitochondrial fractions were studied using DPN as cofactor, and the homogenate and supernatant fractions were studied using TPN as cofactor.

FIGURE 17

THE EFFECT OF NICOTINAMIDE ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE

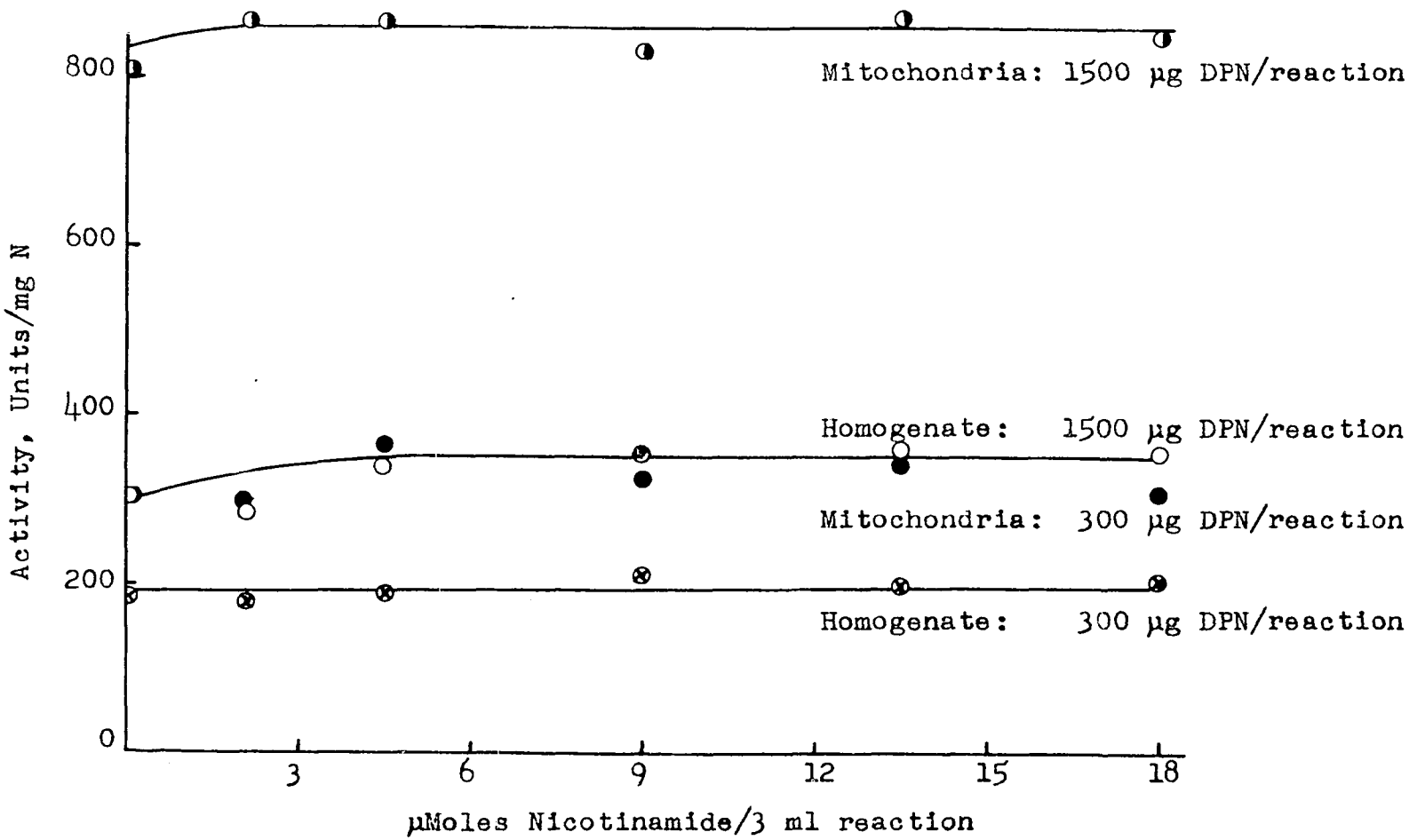


TABLE 15

THE EFFECT OF NICOTINAMIDE ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Nicotinamide μ Moles per reaction	Activity* in Units/mg N			
	Homogenate		Supernatant	
	TPN (100 μg per reaction)	TPN (1000 μg per reaction)	TPN (100 μg per reaction)	TPN (1000 μg per reaction)
0	107	188	134	289
2.1	101	170	124	265
4.5	91	169	126	256
9.0	94	158	127	256
13.5	96	165	121	253
18.0	89	158	131	242

*A unit of activity equals the change in OD of 0.001/minute.

The determinations were made according to the standard procedure described in Chapter II. The sodium pyrophosphate buffer was 0.18 Molar, pH 9.55. A concentration of 3000 μg of DPN or 1000 μg of TPN per reaction was used for the experiments. The total volume of the reaction was kept at 3 ml.

Results. The data showing the effect of substrate concentration upon the rate of oxidation of said substrate are given in Table 16, Figures 19 and 20. The homogenate reaches its maximum activity at about 10 μg of testosterone using the DPN system and the mitochondria behaves

FIGURE 18

THE EFFECT OF NICOTINAMIDE ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE

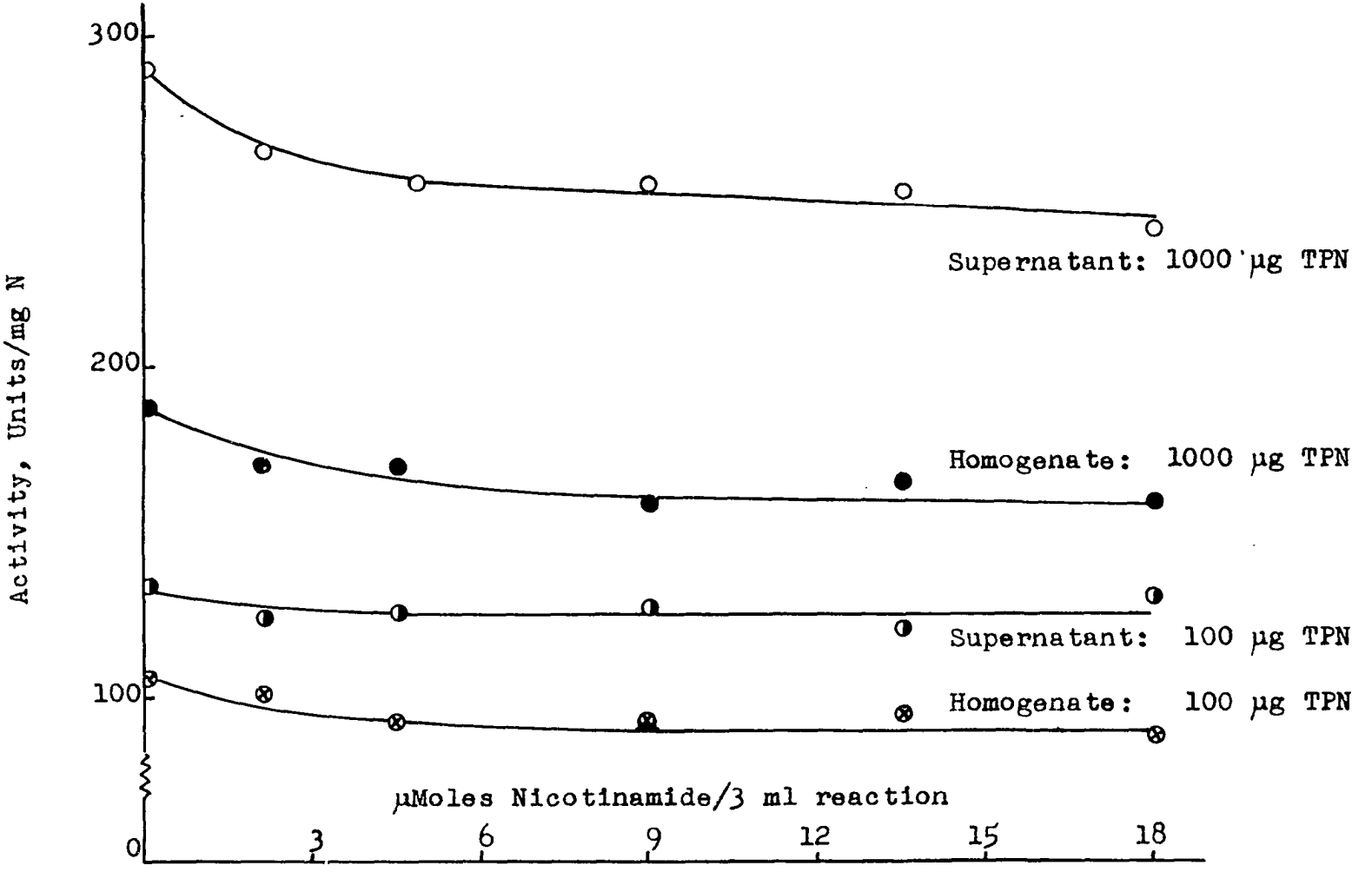


TABLE 16

THE EFFECT OF SUBSTRATE[a] (TESTOSTERONE) CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Testosterone µg per 3 ml reaction	Activity[b] in Units/mg N					
	DPN				TPN	
	Homogenate		Mitochondria		Homog.	Super.
	Prep. 18	Prep. 20	Prep. 18	Prep. 20	Prep. 18	Prep. 18
3.3		250		780		
6.6	300	444	810	1150	59	71
9.8		446		1230		117
13.1	494	457	930	1360	93	128
19.7	447	489	1060	1500	120	193
26.2				1440		
32.8	503	478	1170	1360	184	260
45.9	471	437	1170	1230	222	322
59.0	456	437	1220	1280	243	366

a. The substrate was a water solution of testosterone assaying 32.8 µg/ml.

b. A unit of activity equals a change in OD of 0.001/minute.

FIGURE 19

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE

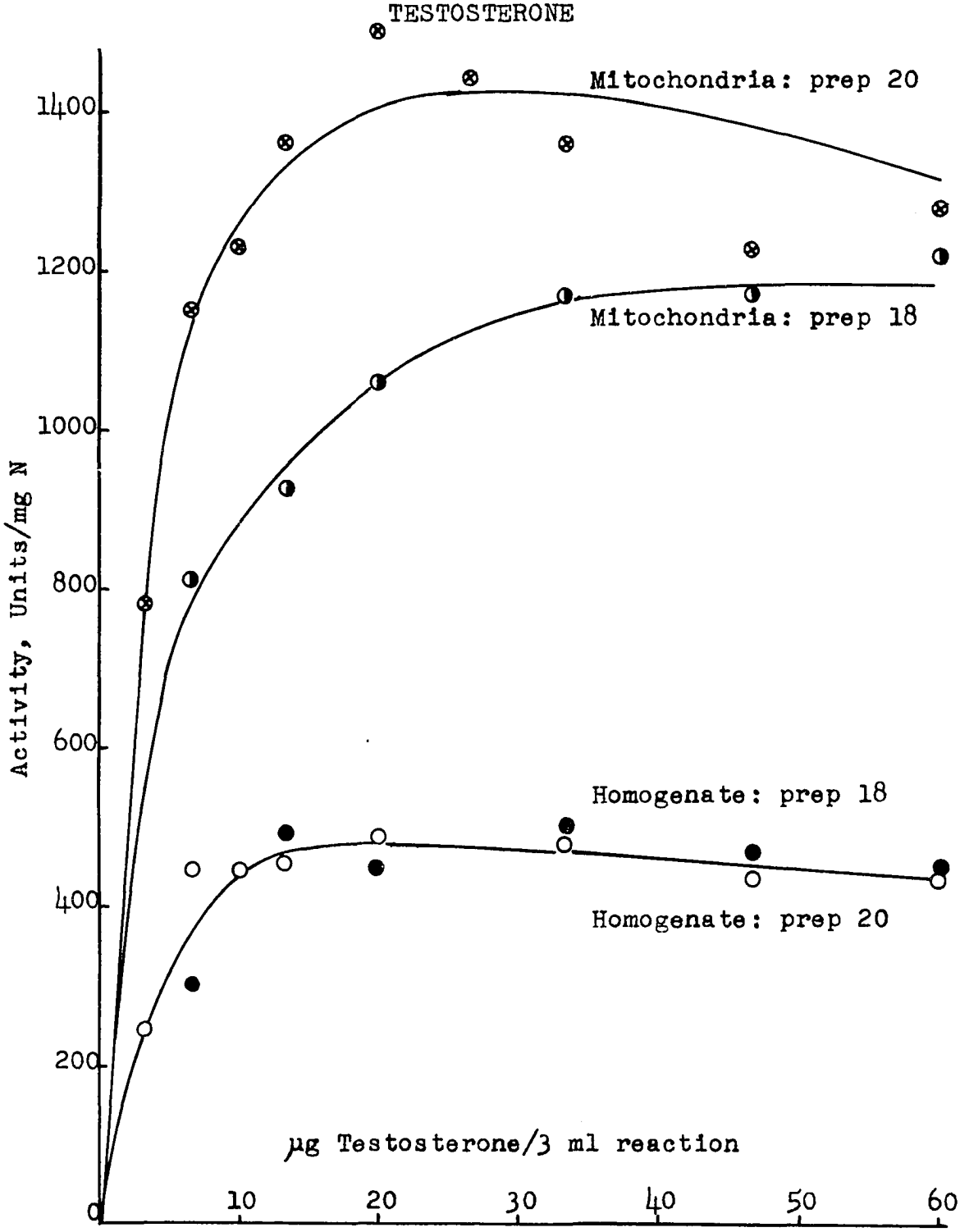
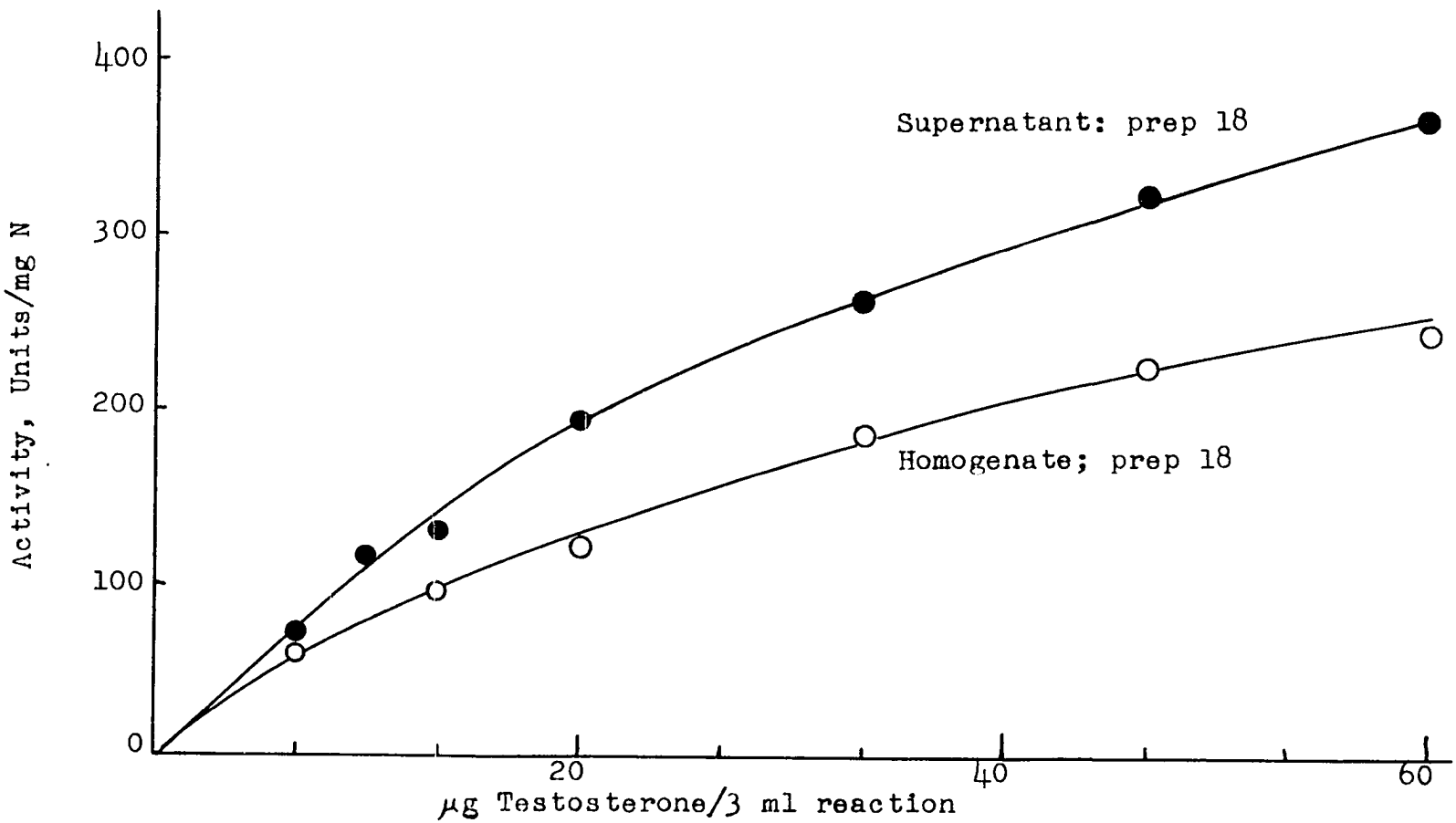


FIGURE 20

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE



in a similar manner (Figure 19). Further increasing of the substrate concentration up to 60 µg did not influence the rate of the reaction in the DPN system.

The concentration of testosterone in the water solutions was not great enough to permit the TPN system using homogenate or supernatant to reach a point at which the concentration would not be limiting the rate of the reaction (Figure 20).

Despite the fact that the substrate concentration using water solutions of substrate was limiting when the TPN reaction system was studied, the water solution of testosterone was used in the test system during the subsequent purification of the TPN linked enzyme activity of the supernatant fraction (Chapter V).

The effect of substrate concentration on the rate of oxidation of said substrate was repeated at a later date using alcohol solutions of testosterone with some of the same tissue fractions used with the water solutions and also with two of the purified fractions of supernatant. These data are considered next.

Alcohol Solutions of Testosterone

Procedure. In order to permit carrying out the experiments at concentrations of substrate greater than 60 µg/reaction, solutions of testosterone were made up in alcohol, at concentrations such that 0.05 ml of alcohol was incorporated in the reaction mixture. This was corrected for by placing 0.05 ml of alcohol in the blank. Thus, any alcohol dehydrogenase present would automatically cancel itself out. However, in practice it was found that while the homogenate contained

alcohol dehydrogenase, the purified TPN fractions did not. Using these alcohol solutions, concentrations of testosterone as high as 300 μg/ reaction were studied.

Results. The effect of substrate concentration on the rate of oxidation of testosterone, using alcohol solutions of testosterone, are given in Table 17 and Figures 21 and 22. The results obtained from the alcohol solutions paralleled the water solutions very closely. Again the DPN system using lyophilized mitochondria reached a maximum rate at a very low concentration of testosterone, namely in this case, about 20 μg/reaction (Figure 21). This is in sharp contrast to the TPN systems using homogenate and purified preparations of supernatant (Figure 22). Two different purified supernatant fractions were studied and in one the maximum rate of reaction using TPN was attained between 70 and 100 μg of testosterone, while in the other preparation and in the homogenate the activity continued to rise even at the highest concentration of testosterone studied, although about 75 per cent of the total reaction rate found had been attained with 100 μg of substrate, which was about 30 per cent of the total concentration of substrate studied.

Thus, in the reactions in which TPN is used as the cofactor, the substrate concentration will be rate limiting to some degree even with the use of alcohol solutions of testosterone.

Summation of DPN + TPN Activity

It was reasoned that if two separate enzymes capable of oxidizing testosterone to 4-androstene-3,17-dione were present in guinea pig liver, one which was specific for DPN as the coenzyme and another which

TABLE 17

THE EFFECT OF SUBSTRATE (TESTOSTERONE) CONCENTRATION ON THE RATE OF OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER

Substrate[a]	Activity[b] in Units / mg N			
	DPN	TPN		
µg per 3 ml	Prep. 36	Prep. 32		Prep. 36
reaction	Lyophil. Mitochondria	Homogenate	Purified TPN fraction	Purified TPN fraction
5	218	12	95	104
10	350	25	168	187
15		31	242	
20	509	44	290	288
30	429	57	367	371
40	493	69	429	438
50	545	77	487	462
60	529	81	521	507
70	616	91	574	474
80	477	92	599	516
90	449	100	621	489
100	568	101	642	535
150	592	121	720	552
200	501	134	781	559
250	501	156	842	538
300	477	157	852	535

a. The substrate was an alcohol solution of testosterone.

b. A unit of activity equals the change in OD of 0.001/minute.

FIGURE 21

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF THE DPN-LINKED OXIDATION OF TESTOSTERONE

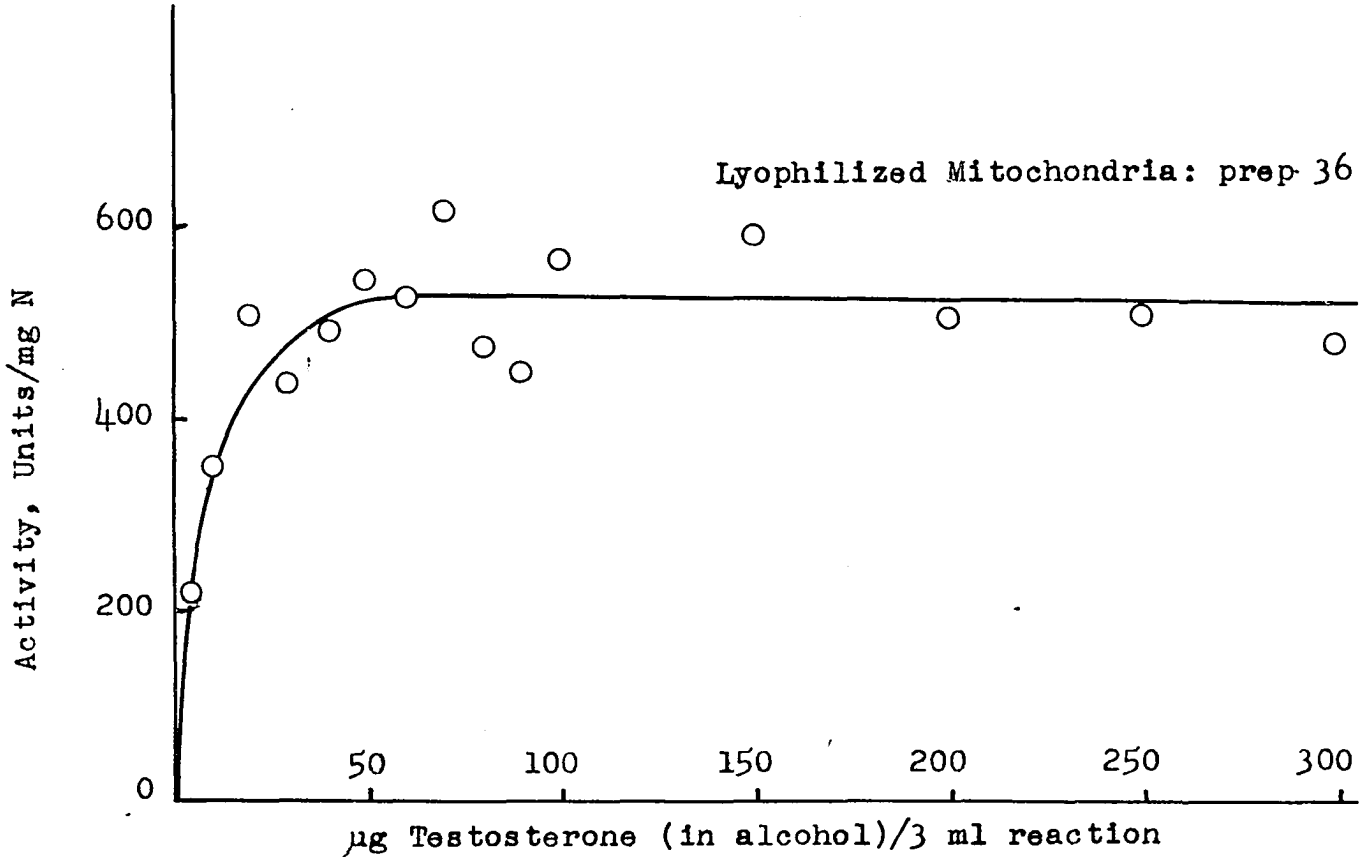
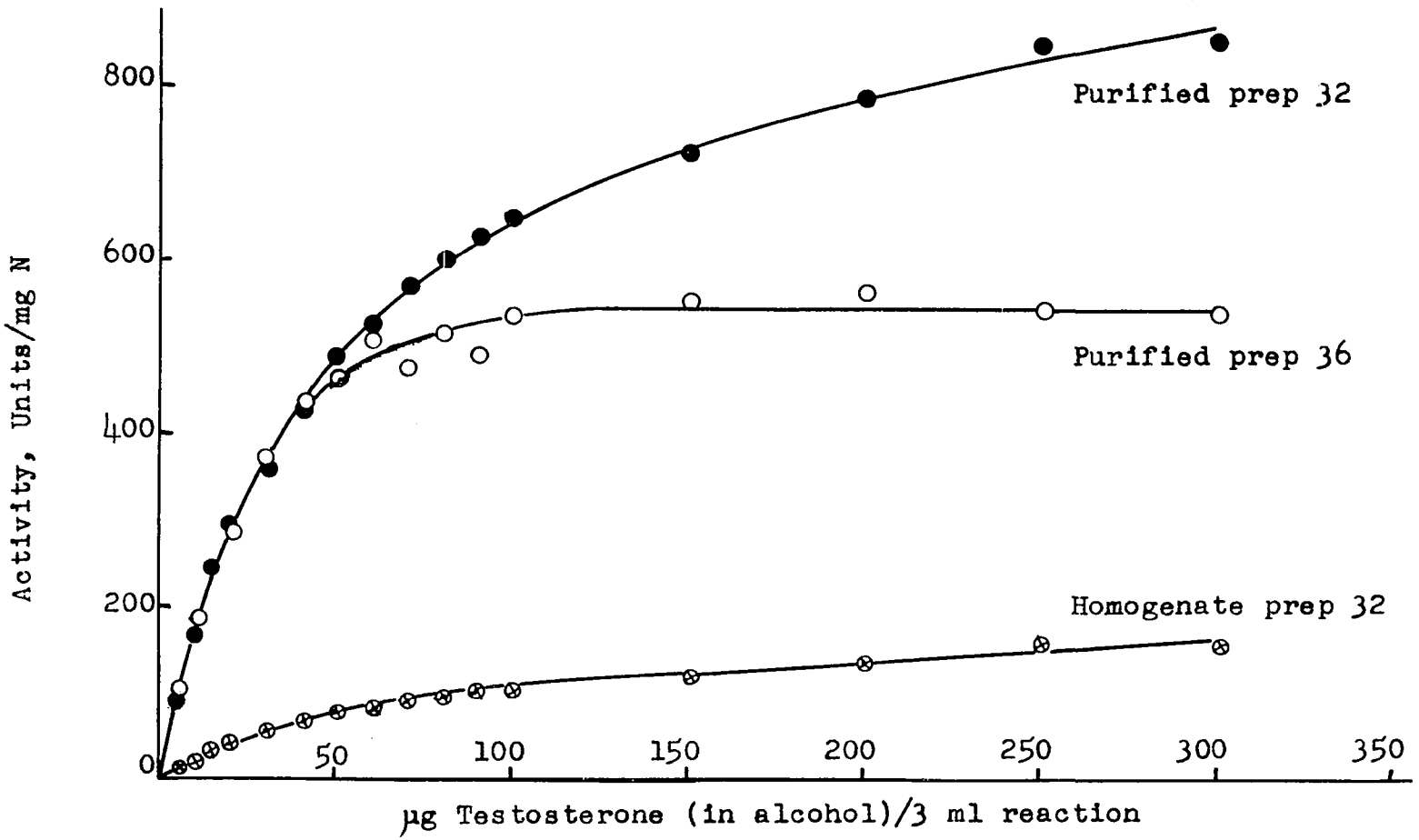


FIGURE 22

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE



was specific for TPN as coenzyme, then the rate of activity of guinea pig liver in a reaction mixture which contained both cofactors should be the summation of the rates of the same liver homogenate with each individual coenzyme. The following experiment was run to test this hypothesis.

Procedure. The rate of oxidation of testosterone as followed by the change in extinction at 3400 Å was determined singly with DPN and TPN as the coenzymes and guinea pig liver homogenate as the enzyme source. The rate of oxidation was subsequently determined in a reaction mixture in which both coenzymes were included. A water solution of testosterone was used as the substrate.

The reaction mixture was as follows: 0.1 ml DPN (15 mg/ml), 0.1 ml TPN (5 mg/ml) or 0.1 ml of each; 1.0 ml of 0.18 M sodium pyrophosphate buffer pH 9.55, 1.8 ml of a water solution of testosterone, and 0.02 ml of 10 per cent guinea pig liver homogenate. A blank was run which contained all reagents except the testosterone substrate.

Results. The results of four separate experiments are tabulated and also averaged in Table 18, and the average of these four experiments is represented graphically in Figure 23. It may be seen in Figure 23 that the rate observed with both TPN and DPN as coenzymes in the reaction mixture is not as high as the sum of the rates obtained when each coenzyme was used singly. The decrease is about 13 per cent. This decrease is not large and may possibly be due to a competition of the enzymes for the same substrate. It will be noted in Figure 20 that a concentration of substrate that was not limiting was never reached for

TABLE 18

SUMMATION OF DPN + TPN ACTIVITY

Experiment	Units Activity* per ml of Guinea Pig Liver Homogenate 10 per cent		
	DPN	TPN	TPN+DPN
1	1325	625	1555
2	970	525	1750
3	1520	530	1685
4	1525	610	1620
AVG.	1335	572	1652

*A unit of activity equals a change of 0.001/minute.

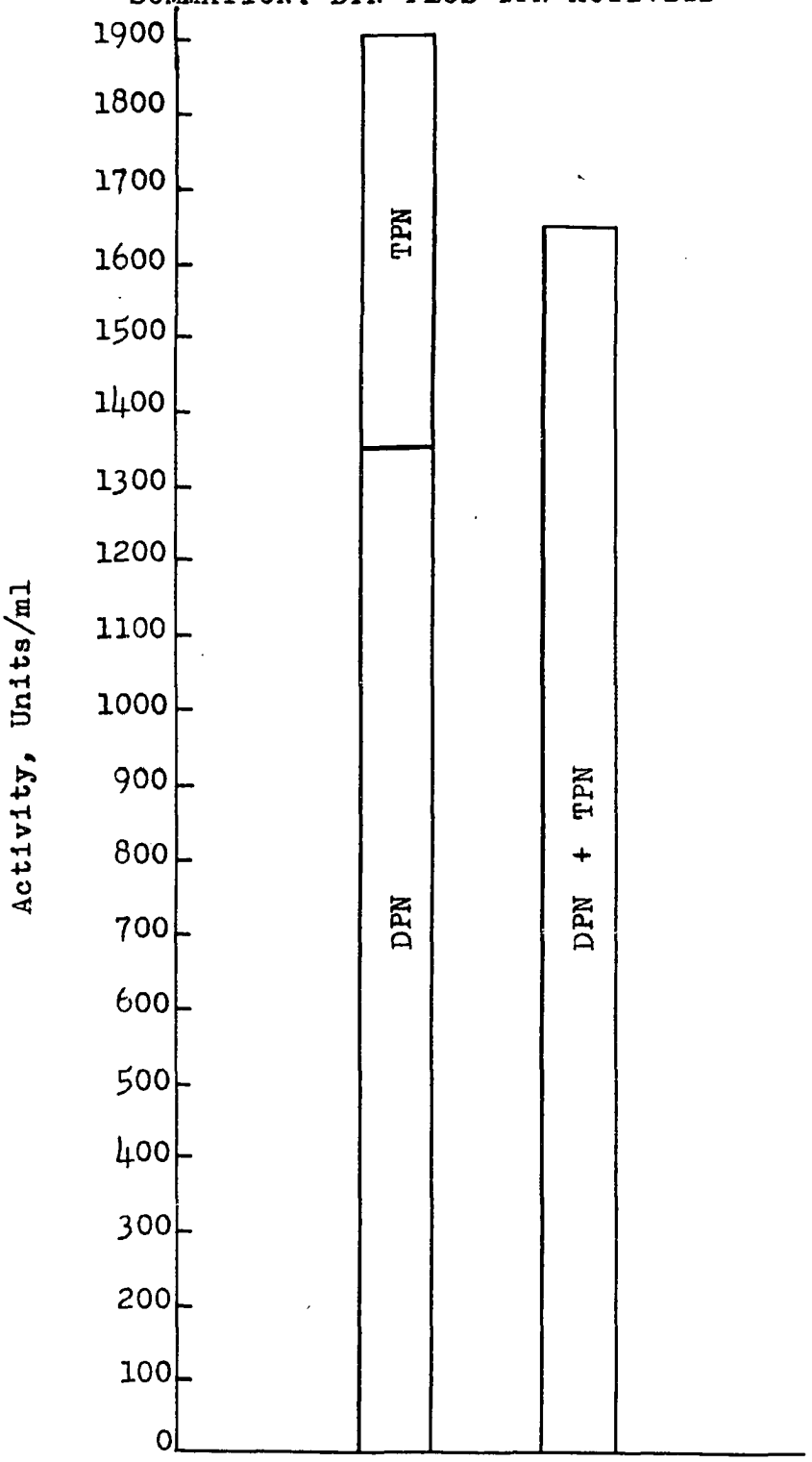
the TPN system and, therefore, a removal of some of the substrate by the DPN system would result in a lowering of the rate of the TPN system when they are both included in the same reaction mixture.

Transhydrogenase Activity

Procedure. Guinea pig liver homogenate and fractions thereof were tested for transhydrogenase activity according to a modified method of Kaplan, et al., (50) in which glucose-6-phosphate (Sigma) and glucose-6-phosphate dehydrogenase (Sigma, 615 Kornberg Units/g) were substituted for isocitrate and isocitrate dehydrogenase as a means of reducing TPN to TPNH. Each one cm cuvette contained 0.25 ml TPN (1 mg/ml), 0.1 ml NaCN (2 mg/ml), 0.1 ml nicotinamide (73 mg/ml), 1.5 ml tris(hydroxymethyl)aminomethane buffer 0.16 M, pH 7.5, 0.2 ml glucose-6-phosphate

FIGURE 23

SUMMATION: DPN PLUS TPN ACTIVITY



dehydrogenase (5 mg/3.5 ml of 0.1 per cent Na_2HCO_3) . The blank cell then received 0.6 ml of water and the experimental cell received 0.6 ml of glucose-6-phosphate (4 mg/ml). To both cells was then added 0.05 ml of the preparation to be tested and water to make the total of 3 ml. After the optical density at 3400 Å became constant (about 6 minutes) 0.2 ml of DPN (2 mg/ml) was added. With active preparations the $E_{3400\ Å}$ increased because of the formation of DPNH from TPNH which was constantly and instantaneously regenerated by the glucose-6-phosphate system.

Results. The results of this experiment are given in Table 19. Also included are transhydrogenase activities of rat heart and guinea pig heart for comparative purposes. The transhydrogenase activity in the fresh tissue homogenates and fractions is also compared with the activity after storage in the cold for 72 hours. Rat and guinea pig heart have the greatest concentration of transhydrogenase, which is approximately four times the concentration in guinea pig liver. The transhydrogenase activity of the guinea pig liver is associated with the mitochondrial fraction and the supernatant 105,400 X G has no transhydrogenase activity. These observations are in accord with those of Humphrey (57) who reports activity in heart and muscle and small activities in liver and kidney tissue. He also reported that this activity is associated with the mitochondrial fraction.

The transhydrogenase enzyme is stable enough to remain active in the tissues after storage for 72 hours in the cold.

The rate of oxidation of testosterone by rat heart homogenate as measured spectrophotometrically at 3400 Å in the usual test system

TABLE 19

TRANSHYDROGENASE ACTIVITY

Tissue	Activity[a]	
	4 hours old	72 hours
Rat heart homogenate	7.54	5.38
Guinea pig heart homogenate	5.18	3.82
Guinea pig liver homogenate	1.50	1.21
Guinea pig liver mitochondria	0.98	0.16
Guinea pig liver soluble[b]	0	0
Guinea pig liver purified supernatant (prep. 36)	0	0

a. Activity expressed as the change in optical density at 3400 Å/10 minutes/ml of 5 per cent tissue (wet weight-volume) after addition of DPN.

b. Supernatant 105,400 X G (2 hours).

using DPN as coenzyme revealed that rat heart had a very low activity. This activity in a 5 per cent preparation was 4 units/ml as compared to guinea pig liver at the same concentration which has approximately 600 units/of activity per ml. Thus high transhydrogenase activity is not necessarily related to a high rate of oxidation of testosterone.

Discussion of Chapter III

The influence of the factors considered in this chapter on the DPN and TPN reactivity of guinea pig liver seem to indicate that two different enzymes are associated with these activities which are both

concerned with the oxidation of testosterone. One of the most striking differences between these two reactivities is manifested under the influence of various hydrogen ion concentrations. Both activities have approximately the same pH optimum, but the DPN activity has a much sharper peak and is denatured at a lower pH than the TPN system. It may also be well to note at this point that the pH optimum of the DPN system at approximately 9.5 is much higher than the pH optima of approximately 7 given in the literature for transhydrogenase reactions (51) and for the DPN isocitric dehydrogenase system reported by Plaut and Sung (52) and by Ville and Gordon (42).

The effect of substrate (testosterone) concentration on these two reactivities is also very different. The DPN activity rises sharply and reaches a maximum activity at a low concentration (10-20 μg/reaction), while the TPN activity rises gradually and almost linearly with substrate concentrations up to 300 μg/reaction. It is impossible at this point to speculate why a dual system should be in operation for the oxidation of this steroid. It should be pointed out, however, that not all of the possible steroids have been investigated as substrates and further investigation may reveal that one of these activities which is of concern here will have a greater affinity for some steroid molecule other than testosterone. The rates of oxidation of some other steroids have been investigated (Chapter IV), but this represents a very small number of these related compounds which can be investigated as substrates. The point to be made here, however, is that the differences which exist between the DPN and TPN activities under the influence

of varying amounts of steroid seems also to point out the fact that these indeed are two different enzymes.

The DPN + TPN summation experiments of this chapter seem also to support the thesis that two different enzymes are concerned with these activities. The activity when DPN and TPN are both included in the reaction mixture was 87 per cent of the sum of the two when the activity was measured singly with each of these coenzymes. Considering that a maximum substrate concentration was not reached for the TPN system, the 13 per cent reduction of rate when both cofactors were included may have been due to competition for substrate as pointed out under the section on the summation experiments.

It is possible that the DPN activity of guinea pig liver is due to a specific TPN enzyme activity and a transhydrogenase as proposed by Talalay and Williams-Ashman (39) with the DPN activity of isocitric dehydrogenase. It could be represented as follows:

$$\text{testosterone} + \text{TPN} \xrightleftharpoons{\text{dehydrogenase}} \text{TPNH} + \text{4-androstenedione} \quad (1)$$

$$\text{TPNH} + \text{DPN} \xrightleftharpoons{\text{transhydrogenase}} \text{DPNH} + \text{TPN} \quad (2)$$

$$\text{Sum: testosterone} + \text{DPN} \rightleftharpoons \text{DPNH} + \text{4-androstenedione}$$

That the enzyme must be a specific TPN enzyme and not DPN (in which case DPN would replace TPN in reaction 1 and reaction 2 would go from right to left) is deduced from the fact that the supernatant fraction still has the ability to oxidize testosterone, but does not exhibit transhydrogenase activity (Table 19). It must also be pointed out that reaction 2 is limited in rate by production of TPNH from reaction 1. In Table 2 it

will be noted that the TPN activity is always approximately half that of the DPN activity and, therefore, it does not seem likely that such a system as expressed above would be operating when DPN activity is noted.

Because these factors point toward the fact that two separate enzymes, one specific for TPN and another for DPN, are responsible for the dual oxidation of testosterone by guinea pig liver, the two activities are treated as such in further work.

CHAPTER IV

CORRELATION OF PRODUCT FORMATION WITH SUBSTRATE DISAPPEARANCE

The establishment of the conditions for the systems for the oxidation of testosterone was followed by an examination of the reaction mixture at various times for the steroid products formed to determine whether the products produced were comparable to the products obtained in equilibria studies with guinea pig liver (48). It also seemed possible that by means of some short term experiments some possible intermediates might be detected. These experiments were conducted using a minimum of tissue and under conditions previously determined for optimum rates as presented in Chapter III.

This qualitative analysis was followed by quantitative experiments to determine whether the rate of change of concentration of DPNH as followed by the change in optical density at 3400 Å was valid as a measure of the rate of conversion of testosterone to 4-androstene-3, 17-dione. These qualitative and quantitative aspects of the reaction are presented in this chapter.

Qualitative Determination of Products

Procedure. Six 93 ml reaction mixtures were incubated with guinea pig liver tissue to determine what products were formed from

testosterone by these guinea pig liver fractions. A quantity of 1.55 ml of 10 per cent (wet weight-volume) liver homogenate, mitochondria and supernatant were incubated with the DPN system, while 3.1 ml of those fractions were incubated with the TPN system. The reaction mixtures used were as follows: 55.8 ml of a water solution of testosterone (31.8 µg/ml), 31 ml of 0.18 M sodium pyrophosphate buffer pH 9.50, 93 mg of DPN or 46.5 mgs TPN, enzyme as indicated and water to bring the total volume to 93 ml.

The reaction mixtures were incubated at 37°C and 15 ml samples were removed at 0, 5, 10, 15, 20 and 30 minute intervals and immediately extracted 3 times with an equal quantity of ether. These ether extracts were combined and evaporated to dryness and the total residue from each was subjected to paper chromatography by the method of Kochakian and Stidworthy (53) using propylene glycol as the stationary phase and a 1-1 mixture of cyclohexane-benzene as the mobile phase. A standard which contained equimolar amounts of testosterone, epitestosterone, androsterone, 4-androstenedione and androstanedione, was applied to each paper. After development the papers were dried, scanned by ultra violet light (54) and then sprayed with an equal mixture of 2 per cent m-dinitrobenzene in alcohol and 20 per cent KOH in alcohol. The papers were studied carefully to determine all metabolites formed and also to estimate the amount of conversion.

Results. The results of these experiments are tabulated in Tables 20 and 21. Table 20 contains the results of experiments in which DPN was used as a coenzyme and Table 21 contains the results in which TPN was used as a coenzyme.

TABLE 20

CHROMATOGRAPHIC DETERMINATION OF PRODUCTS FORMED BY THE DPN-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER FRACTIONS

Time	Homogenate				Mitochondria				Supernatant			
Min.	UV		MDB		UV		MDB		UV		MDB	
	T	A	T	A	T	A	T	A	T	A	T	A
0	++++	+-	++++	+-	++++	+-	++++	+-	Increase in androstenedione spot, but decrease in testosterone hardly observable.			
5	+++	+	+++	+	+++	+	+++	+				
10	++	++	++	++	++	++	++	++				
15	+	+++	+	+++	+	+++	+	+++				
20	+-	++++	-	++++	+-	++++	-	++++				
30	-	++++	-	++++	-	++++	-	++++				

KEY (Estimation of Amount of Steroid)

-	None
+--	Very faint
+-	Faint
+	
++	
+++	
++++	Maximum

UV. Steroid area detected by ultraviolet absorption.

MDB. Steroid area detected by spraying paper with m-dimitrobenzene.

T. Area detected with mobility comparing to that of testosterone

A. Area detected with mobility comparing to that of 4-androstene-3,17-dione.

TABLE 21

CHROMATOGRAPHIC DETERMINATION OF PRODUCTS FORMED BY THE TPN-LINKED OXIDATION OF TESTOSTERONE BY GUINEA PIG LIVER FRACTIONS

Time	Homogenate				Supernatant				Mitochondria			
Min.	UV		MDB		UV		MDB		UV		MDB	
	T	A	T	A	T	A	T	A	T	A	T	A
0	++++	+-	++++	-	++++	+--	++++	+--	++++	-	++++	-
5	+++	+	+++	+	+++	+	+++	+	++++	+--	++++	+--
10	++	++	++	++	++	++	++	++	++++	+-	++++	+-
15	+	+++	+-	+++	+	+++	+	+++	+++	+	+++	+
20	+-	+++	+--	++++	+-	++++	+-	++++	+++	+	+++	+
30	+-	++++	+---	++++	+-	++++	+--	++++	+++	+	+++	+

KEY (Estimation of Amount of Steroid)

-	None
+--	Very faint
+-	Faint
+	
++	
+++	
++++	Maximum

UV. Steroid area detected by ultraviolet absorption.

MDB. Steroid area detected by spraying paper with m-dinitrobenzene.

T. Area detected with mobility comparing to that of testosterone.

A. Area detected with mobility comparing to that of 4-androstene-3,17-dione.

It will be noted that a compound with the same mobility as 4-androstene-3,17-dione was the only metabolite detected with any of the tissue fractions and with either cofactor. The testosterone was completely converted to 4-androstene-3,17-dione in 30 minutes if DPN was used as the cofactor and either the liver homogenate or the mitochondria fraction was used as the source of enzyme. The same reactions were very nearly complete in 30 minutes when homogenate and supernatant were used as the enzyme source with TPN as the cofactor. It will also be noted that there was some conversion of testosterone when the supernatant fraction was used as enzyme source and DPN as cofactor and also when the mitochondrial fraction was used as the enzyme source with TPN as the cofactor. This incomplete separation of enzyme activity is also borne out in the rate experiments which show some DPN activity in the supernatant and some TPN activity in the mitochondrial fraction.

Correlation of DPNH Formation With Substrate Disappearance and Product Appearance

Procedure. Guinea pig liver mitochondria (1 ml of 10 percent) was incubated at 37°C with the following reaction mixture; 60 ml of a water solution of testosterone (32.6 µg/ml), 30 ml of 0.18 M sodium pyrophosphate buffer pH 9.5 and 90 mgs of DPN. Another identical reaction was made in which water was substituted for the testosterone solution for the purpose of measuring an endogenous rate.

The reaction mixtures without enzyme were brought to 37°C in a water bath. At zero time 1 ml of enzyme was added to both reactions and 3 ml each of the endogenous mixture and the reaction mixture were placed in their respective 3 ml cuvettes in the Cary spectrophotometer at 37°C

and the increase in optical density (OD) was followed at 3400 Å. One 10 ml sample was removed from the reaction mixture before addition of the enzyme and more 10 ml samples were removed immediately after addition of enzyme and at three to four minute intervals thereafter until the reaction was complete as indicated by no further change in OD in the spectrophotometer. Two ten ml samples were also removed from the endogenous reaction, one immediately after the tissue was added and another at the termination of the experiment. All ten ml samples removed from the reaction mixtures were pipetted immediately into 50 ml of warm ethyl alcohol to stop any further reaction by precipitation of tissue proteins. The alcohol was evaporated from the samples under reduced pressure at 70°C. The water residues were then extracted for 1 hour with ethyl ether in a liquid-liquid continuous flow extractor. The ether extracts were evaporated to dryness and the total residue was applied to paper chromatograms for separation of the steroid products (method of Kochakian and Stidworthy (53)). After development the papers were dried and the testosterone and 4-androstenedione areas were located by scanning with ultraviolet light (54). Equal areas containing the steroid were then cut from the paper and eluted with acetone. The acetone was evaporated and the amounts of testosterone and 4-androstene-3,17-dione were determined spectrophotometrically in ethyl alcohol. The molar extinction for testosterone at 2410 Å in ethyl alcohol is 15,800 and for androstenedione at 2400 Å in ethyl alcohol is 17,170 (55).

Results. The results of this experiment are shown in Table 22 and Figure 24. The amount of DPN which was reduced to DPNH at various times was calculated from the optical density readings at 3400 Å using

TABLE 22

QUANTITATIVE CORRELATION OF PRODUCTS

Time in Min.	Theoretical Androstenedione calculated from DPNH μg	Testosterone metabolized μg	Androstenedione formed μg
0[a]			3
0[b]	0	31	4
3	50	90	42
4	69	109	---
6	102	139	92
7	119	151	88
9	150	166	130
12	186	193	160
17	199	204	167
18	203	194	187

Level of testosterone at 0 time before tissue was 209 μg.

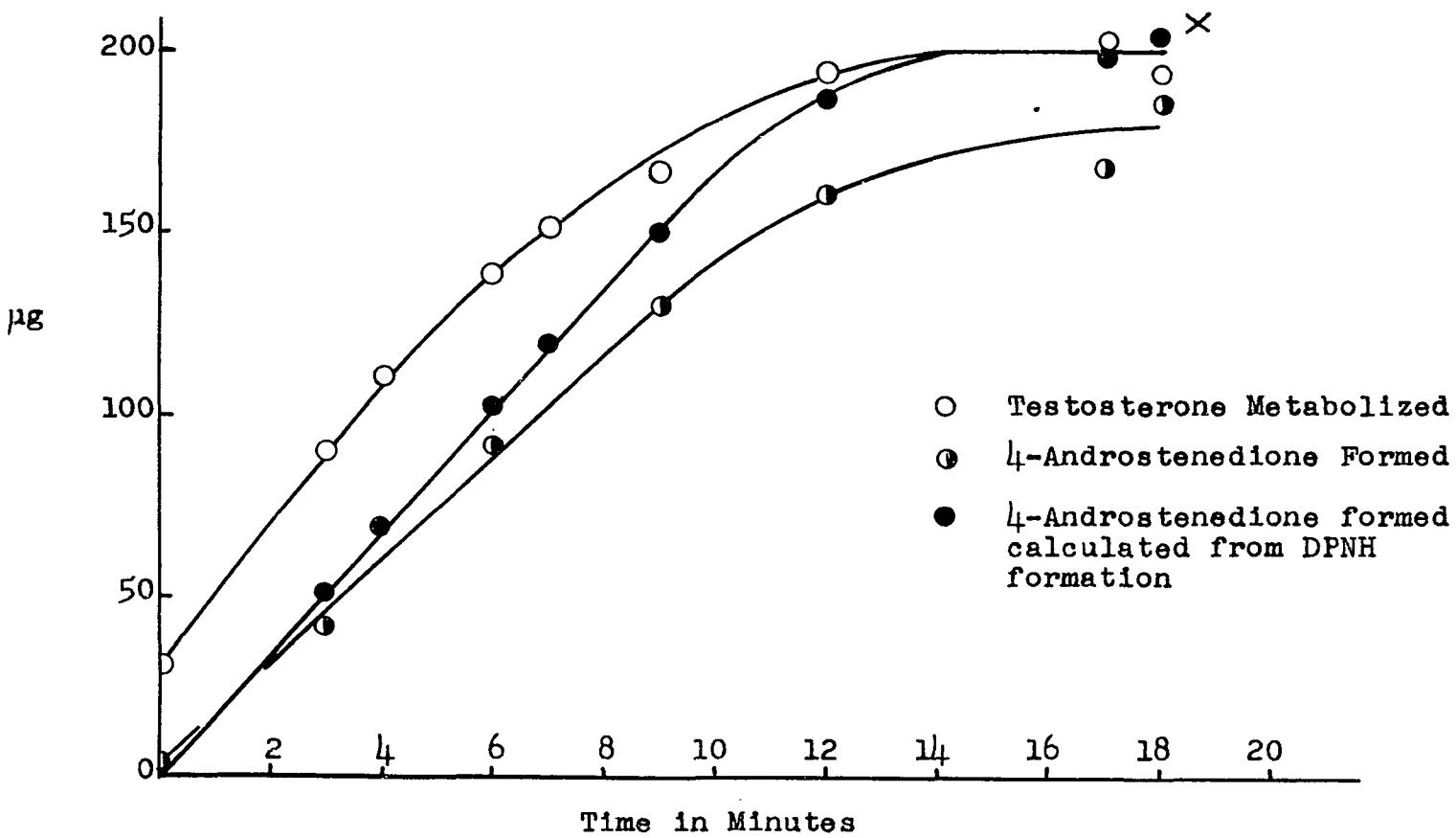
a. Before addition of tissue.

b. Sample removed immediately after addition of tissue.

6.22×10^3 as the molar $E_{max.}$ for DPNH. This amount of DPNH was converted to μg amounts of 4-androstene-3,17-dione assuming a mole to mole stoichiometric relationship. This amount of 4-androstenedione is plotted in Figure 22 along with the actual amount of testosterone metabolized and the amount of 4-androstene-3,17-dione formed as isolated and determined by their extinction in alcohol.

FIGURE 24

QUANTITATIVE CORRELATION OF SUBSTRATE DISAPPEARANCE WITH PRODUCT FORMATION



It will be noted that immediately after addition of the tissue and for a time up to seven minutes the testosterone which was metabolized or which disappeared from the reaction mixture is approximately 30 μg less than the theoretical as calculated from the DPNH formed. After seven minutes, however, the testosterone metabolism approaches the theoretical and becomes the same as the reaction approaches and reaches completion. This amount at completion agrees favorably with the amount of testosterone which was determined to be in the reaction before addition of the tissue (amount represented in Figure 24 by x). This discrepancy between the amount of testosterone metabolized and that amount indicated by DPNH formation may indicate the formation of an enzyme-substrate complex which is stable enough not to be broken in alcohol solution and either is not extracted with ether or does not chromatograph at the same rate as testosterone so that it was not determined with the testosterone. It was observed that a small ultraviolet positive spot appears on papers when the ether extracts of the reaction mixtures are chromatographed. It has a smaller R_f than testosterone but did not correspond to any uv positive spot reported (53). The uv positive material in this area was not examined further.

It also will be noted in Figure 22 that the amount of 4-androstene-3,17-dione formed agrees very well with the theoretical amount calculated from DPNH formation at the beginning of the reaction but becomes progressively less as the reaction nears completion. This decrease is of the order of 10 per cent and may represent conversion to some other product even though no other product was detected in previous experiments (Table 18).

Despite these discrepancies discussed above, it was concluded that the change in extinction at 3400 Å was largely a measure of the reaction

testosterone + DPN $\rightleftharpoons$ DPNH + 4-androstene-3,17-dione

and not of some other side reaction and, therefore, is a valid method for the measurement of the rate of this reaction.

CHAPTER V

FURTHER PURIFICATION OF THE TPN SPECIFIC COMPONENT

After characterization of the TPN and DPN specific reactions in the mitochondria and supernatant fractions of guinea pig liver, it seemed necessary to further fractionate and purify these components for further study.

A comparison of the activity (TPN as coenzyme) in homogenate and supernatant 105,400 X G of tissue preparations used for the remainder of the dissertation is given in Table 23. Purification methods considered in this chapter are all methods concerned with the further purification of the activity in the supernatant fraction of guinea pig liver.

Centrifugation at Higher Gravity

Centrifugation at 105,400 X G

Procedure. Seven grams of guinea pig liver were homogenized in 70 ml of cold 0.25 M sucrose in the Virtis for 1 minute with the dial set at 30 and for another 15 seconds with the dial set at 50. A portion of the homogenate was removed for testing and the remainder was centrifuged at 1500 X G for 30 minutes at 0°C. The sediment was discarded and the supernatant was centrifuged at 24,500 X G for 1 hour at 0°C. The sediment was suspended in the original volume of sucrose and the

TABLE 23

GUINEA PIG LIVER ENZYME PREPARATIONS

Prep #	Homogenate			Supernatant 105,400 X G		
		TPN			TPN	
	mg N	Activity*		mg N	Activity*	
	ml	per ml	per mg N	ml	per ml	per mg N
23	3.32	442	133	1.72	426	249
24	6.52	1619	248	3.42	1407	411
25	5.77	1162	201	3.49	1096	313
26	lost	1326		3.40	1148	338
27		1372		3.31		
28				3.28	1232	376
29	7.01			3.48	1220	351
30	2.88	500	174	1.80	536	298
31	6.15	1280	208			
32	6.26	950	152			
33	6.17	830	135	4.14	840	203
34					930	
35	6.69	1120	167	3.47	1030	297
36	6.68	875	131	3.88	895	231
37				3.65	954	261

*. The activity is expressed as Units (change in optical density of 0.001 per minute).

supernatant, after removal of a small portion, was centrifuged at 105,400 X G for 1 hour at 4°C. The sediment was again suspended in the original volume of 0.25 M sucrose. Enzyme activities using systems employing both DPN or TPN as cofactors and nitrogen content were determined on each of these fractions.

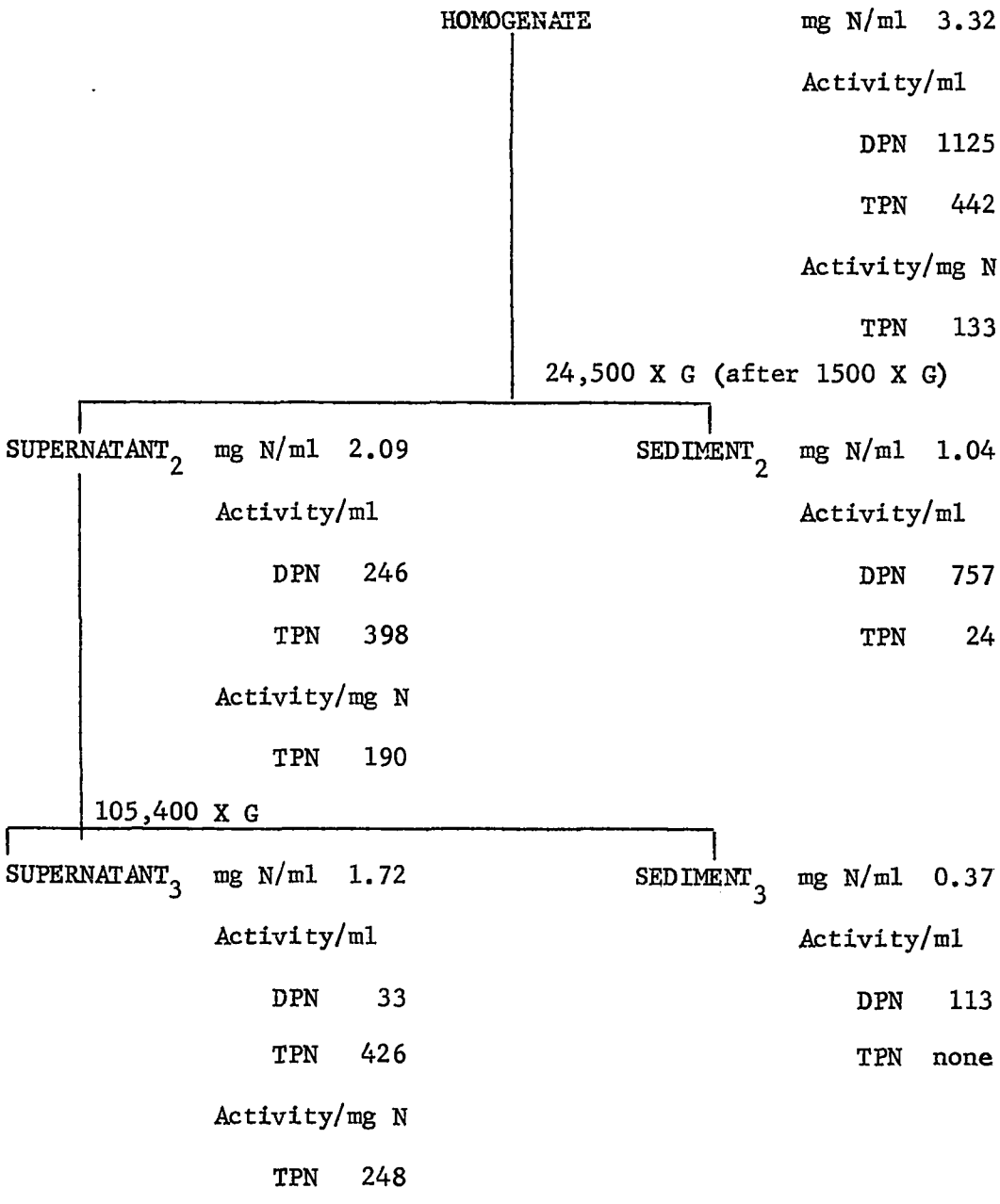
Results. In Figure 25 is shown the diagramatic separation and the results obtained. It is apparent that no enzyme activity is lost in the TPN system in the process of this separation, and it may be assumed that the enzyme responsible for the oxidation of testosterone using TPN as the coenzyme is a truly soluble component and not associated with the particulate components of the cell. There is very little of the DPN specific component in this soluble fraction since it contains about 3 per cent of the original activity of the homogenate. The purification of the TPN specific component is approximately 2-fold on an activity/mg N basis.

A similar experiment was carried out in an identical manner except that water was used as the homogenizing and separating medium. The results of the experiment are recorded in Figure 26. The amount of enzyme activity with TPN as cofactor compared to that separated in sucrose does not appear to be altered. It is apparent, however, that more nitrogen was centrifuged out at 105,400 X G using sucrose as the medium than when water was used as the medium. Also, complete separation of the DPN component is not as good. Thus, the degree of purification of the TPN component on an activity/mg N is only about 1.5 fold. Therefore, it seemed best to use sucrose as a fractionating medium whenever possible.

FIGURE 25

FRACTIONATION OF PREP 23

Homogenization at 10 per cent in Virtis
in 0.25 M Sucrose



The activity is expressed as Units
(change in optical density of 0.001 per minute)

FIGURE 26

FRACTIONATION OF PREP 23

Homogenization at 10 per cent in Virtis in Water

HOMOGENATE

mg N/ml 3.29

Activity/ml

DPN 986

TPN 404

Activity/mg N

TPN 123

24,500 X G (after 1500 X G)

$SUPERNATANT_2$	$SEDIMENT_2$
mg N/ml 2.68	mg N/ml 1.21
Activity/ml	Activity/ml
DPN 260	DPN 917
TPN 304	TPN 88
Activity/mg N	
TPN 190	

105,400 X G

$SUPERNATANT_3$	$SEDIMENT_3$
mg N/ml 2.17	mg N/ml 0.40
Activity/ml	Activity/ml
DPN 127	DPN 106
TPN 420	TPN none
Activity/mg N	
TPN 194	

The activity is expressed as Units
(change in optical density of 0.001 per minute)

Comparison of Centrifugation at 105,400 X G for 1 and 3 Hours

Procedure. Another experiment was performed to determine whether centrifugation for a longer period of time at 105,400 X G would have an effect on the TPN active component and the nitrogen content. Thus, guinea pig liver was homogenized at a concentration of 25 per cent in the Virtis in a similar manner to that outlined above. The homogenate was centrifuged at 1500 X G for 30 minutes at 0°C and the supernatant was decanted. This supernatant was then centrifuged at 24,500 X G for 1 hour at 0°C and the supernatant again decanted. A portion of this supernatant was centrifuged at 105,400 X G for 1 hour at 4°C, while the remainder was centrifuged under the same conditions for 3 hours.

Results. In Table 24 are summarized the results of this separation. No significant decrease was noted in the enzyme activity, and very little decrease in nitrogen content was noted. The usual procedure in following experiments was to centrifuge for 2 hours.

TABLE 24

COMPARISON OF CENTRIFUGATION AT 105,400 X G FOR 1 AND 3 HOURS

	Units Act/ml	Mg N/ml	Units Act/Mg N
Supernatant 105,400 X G for 1 hour	1407	3.42	411
Supernatant 105,400 X G for 3 hours	1355	2.87	472

A unit of activity equals a change in OD of 0.001/min.

Effect of Pre-centrifugation vs. No Pre-centrifugation at 24,500 X G

Procedure. In order to facilitate a faster separation and gain the TPN active supernatant component, an experiment was run to determine if centrifugation at 24,500 X G had any effect on the subsequent separation at 105,400 X G. In this case a portion of homogenate (25 per cent wet weight) after centrifugation at 1500 X G for 30 minutes was centrifuged for 1 hour at 24,500 X G and then the supernatant centrifuged at 105,400 X G for 1 hour. Another portion of the homogenate was fractionated directly at 105,400 X G for 1 hour.

Results. The activity and nitrogen content of the supernatant of the two fractions thus treated are shown in Table 25. No differences were noted; so the centrifugation at 24,500 X G was dispensed with in any case when the mitochondrial fraction was not needed.

TABLE 25

THE EFFECT OF CENTRIFUGATION AT 24,500 X G ON THE ACTIVITY OF SUPERNATANT OF 105,400 X G

	Units Act/ml	Units Act/mg N
Supernatant 105,400 X G 1 hour		
previously spun at 24,500 X G for 1 hour	889	350
not spun at 24,500 X G	886	349

A unit of activity equals the change in OD of 0.001/min.

Ammonium Sulfate Fractionation of Supernatant

Activity of Supernatant from 0-70 Per Cent Saturation

Procedure. Seven 10 ml aliquots of supernatants 105,400 X G (25 per cent wet weight) of guinea pig liver were treated with varying amounts of ammonium sulfate from 0 to 0.5 g/ml. The tissue was allowed to stand in the cold in the salt solution for approximately 2 hours, after which time the aliquots were centrifuged at 24,500 X G for 1 hour at 0°C. The supernatants from the centrifugation were placed in individual dialyses bags and dialyzed against cold 0.1 M sodium phosphate buffer pH 7.4 for 1 hour with changes every half hour. Dialysis was continued against 0.2 M sodium phosphate buffer pH 7.4 for 16 hours with one change after 1 1/2 hours. Some increase in volume had occurred, so all samples were diluted to 15 ml and the enzyme activity using TPN as cofactor and the nitrogen content were determined on each sample. The supernatant to which no ammonium sulphate was added was not dialyzed but was diluted to 15 ml before the enzyme activity and nitrogen content were determined.

Results. In Table 26 are given the results of this experiment. Increasing amounts of ammonium sulfate precipitated the protein enzyme until it was all precipitated at 70 per cent saturation. It also may be noted that some degree of purification was achieved since the activity/mg N of the supernatant was 1.97 in fraction 1 and had risen to 3.46 in fraction 5.

The sediments from fractions 6 and 7 were redissolved in 10 ml of 0.25 M sucrose and the enzyme activity was determined without dialysis.

TABLE 26

AMMONIUM SULFATE FRACTIONATION

Fraction	$(NH_4)_2SO_4$		Activity Units per ml	Mg N per ml	Activity Units per mg N
	gr/ml	% sat.			
1*	0	0	460	2.33	197
2	0.05	7	404	2.08	196
3	0.10	14	387	1.84	210
4	0.20	28.5	352	1.30	271
5	0.30	43	284	0.82	346
6	0.40	55.5	16	0.42	4
7	0.50	70	0	0.25	
6 sediment*			269		
7 sediment*			287		

* This fraction was not dialyzed

A unit of activity equals a change in OD of 0.001/min.

The activity of these sediments given in the table has been corrected to a 15 ml volume so that it can be compared with other data there. The activity of the sediment is less than the untreated supernatant, which may be due to the inactivation or inhibition by salt concentration since these two fractions were not dialyzed.

Closer Fractionation of 35-55 Per Cent Saturation

Procedure. In order to narrow the range in which the best purification could be gained, a second ammonium sulfate fractionation was performed in which the concentration of $(NH_4)_2SO_4$/ml was increased in

0.05 g increments instead of the 0.1 g increment of the previous experiment.

Five 10 ml aliquots of guinea pig liver supernatant 105,400 X G were treated with increasing amounts of $(NH_4)_2SO_4$ from 0.25 to 0.4 grams/ml in 0.05 g increments/ml. The tissue was allowed to stand in the cold salt solution for 1 hour followed by centrifugation at 24,500 X G for 1 hour at 0^oC. The supernatants from this centrifugation were dialyzed against a solution of 0.2 M potassium phosphate pH 7.0 and 0.25 M sucrose and 10 per cent polyvinylpyrrolidone for 2 hours. This was followed by two 1 hour periods against 0.2 M potassium phosphate pH 7.0, and then they were dialyzed against 0.2 M potassium phosphate overnight. After dialysis all fractions were diluted to 16 ml. The sediments from each of the fractions were redissolved in 10 ml of 0.25 M sucrose.

Results. The results of the experiment are given in Table 27. All values given in the activity/ml column have been corrected to the total volume of 16 ml. It will be noted that the precipitated material of the two fractions 43 to 55 per cent saturation would contain 65 per cent of the total enzyme activity while retaining 45 per cent of the nitrogen. Also, the activity of the sediment plus supernatant in each case except fraction 5 gives as much activity as the untreated supernatant. This is a good indication that no inactivation is caused by the ammonium sulfate.

Balance on All Fractions 43-55 Per Cent Saturation

After determining the concentration of ammonium sulfate at which the enzyme was precipitated, the next step was to precipitate the least

TABLE 27

FURTHER AMMONIUM SULFATE FRACTIONATION

Fraction	$(NH_4)_2SO_4$		Activity* Units per ml	Mg N per ml	Activity* Units per mg N	Per Cent of Total Activity
	g/ml	% Sat				
			Supernatant			
1	0	0	540	1.84	293	100
2	0.25	35.5	536	1.33	403	99
3	0.30	43	438	0.82	534	81
4	0.35	49.5	272	0.50	544	50
5	0.40	55.5	94	0.19	494	16
			Sediment (not dialyzed)			
2			42			7
3			152			28
4			278			51
5			350			65

* A unit of activity equals a change in OD of 0.001 per minute.

active protein and then to increase the concentration of $(NH_4)_2SO_4$ in the supernatant in order to precipitate the enzyme-rich fraction.

Procedure. Four 10 ml samples of guinea pig liver supernatant 105,400 X G were treated with varying amounts of ammonium sulfate as indicated in Table 28 in the grams of $(NH_4)_2SO_4$ initial column. They were allowed to stand in the cold for one hour and then were centrifuged at 24,500 X G for 1 hour. The sediments from these fractions were

TABLE 28

AMMONIUM SULFATE FRACTIONATION BALANCE SHEET

Fraction	Designation	Grams of $(NH_4)_2SO_4$/ml			mg N per ml	Activity* Units per ml	Per Cent of Total Activity	Activity* Units per mg N
		Initial	Increment	Total				
1		0	0	0	1.79	632	100	353
2	Super.	0.30	0.05	0.35	0.48	284	45	592
3	Super.	0.3	0.1	0.4	0.32	92	15	288
4	Super.	0.35	0.05	0.4	0.28	110	17	392
5	$Sed._1$	0.3	0.1	0.4	1.07	162	25	151
6	$Sed._1$	0.3	0.05	0.35	1.10	168	26	153
7	$Sed._1$	0.35	0.05	0.4	1.51	348	55	230
8	$Sed._2$	0.3	0.1	0.4	0.46	328	52	704
9	$Sed._2$	0.3	0.05	0.35	0.27	128	20	474
10	$Sed._2$	0.35	0.05	0.4	0.12	76	12	633

* A unit of activity equals a change in OD of 0.001/min.

suspended in 0.25 M sucrose and given the designation of $Sediment_1$ according to the appropriate concentration of ammonium sulfate noted in Table 28. The supernatants were treated with increasing amounts of ammonium sulfate as indicated in the increment column. These were again allowed to stand for 1 hour in the cold, followed by centrifugation at 24,500 X G for 1 hour at $0^{o}C$. The sediments from this centrifugation were redissolved in 0.25 M sucrose and designated as $Sediment_2$. The supernatants from this centrifugation are called supernatant in Table 28.

All of the fractions, ten in all including a 10 ml sample which had not been treated with $(NH_4)_2SO_4$, were dialyzed against 0.2 M potassium phosphate pH 7.4 for approximately 18 hours with 6 changes during the first six hours and 2 during the last hour. After dialysis all fractions were diluted to 15 ml and the nitrogen content and enzyme activity using TPN as cofactor were determined.

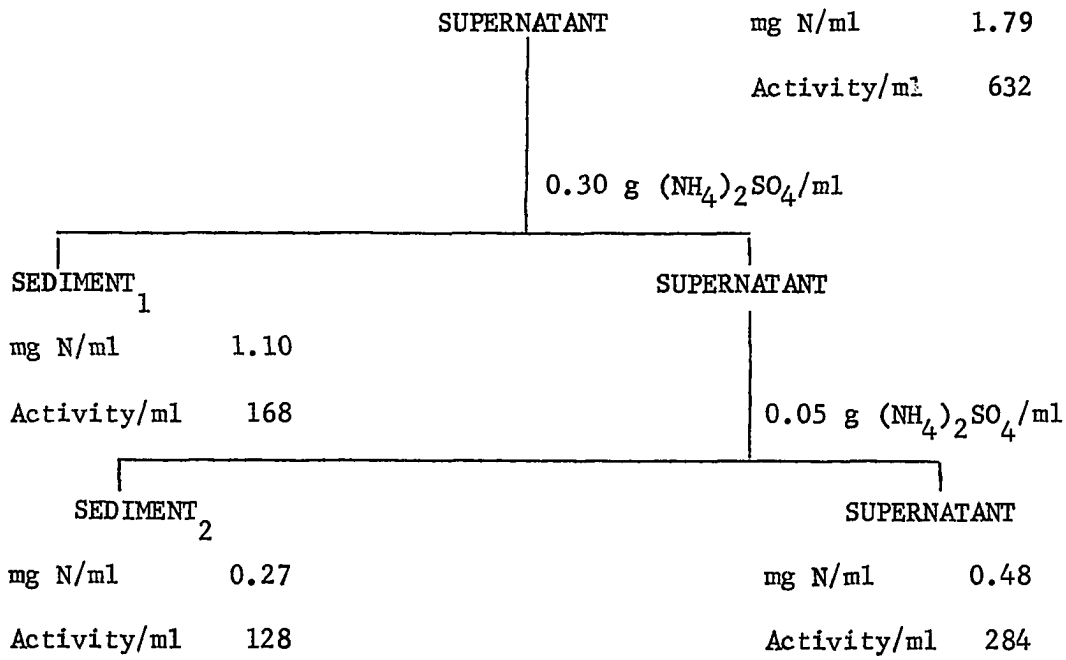
Results. The results are shown in Table 28 and Figures 27, 28 and 29. These data show that the greatest purification is achieved with the $Sediment_2$ 0.3 to 0.4 g $(NH_4)_2SO_4$/ml fraction. This nearly two-fold purification is gained only with the loss of approximately one-half of the total enzyme activity. This fraction was used for most of the subsequent purification work.

It may be noted from Figures 27, 28 and 29 that recovery of nitrogen and enzyme activity was good, indicating again little denaturation during the purification.

FIGURE 27

AMMONIUM SULFATE FRACTIONATION

$0.30 - 0.35$ g $(NH_4)_2SO_4$/ml Fractionation



SUMMATION

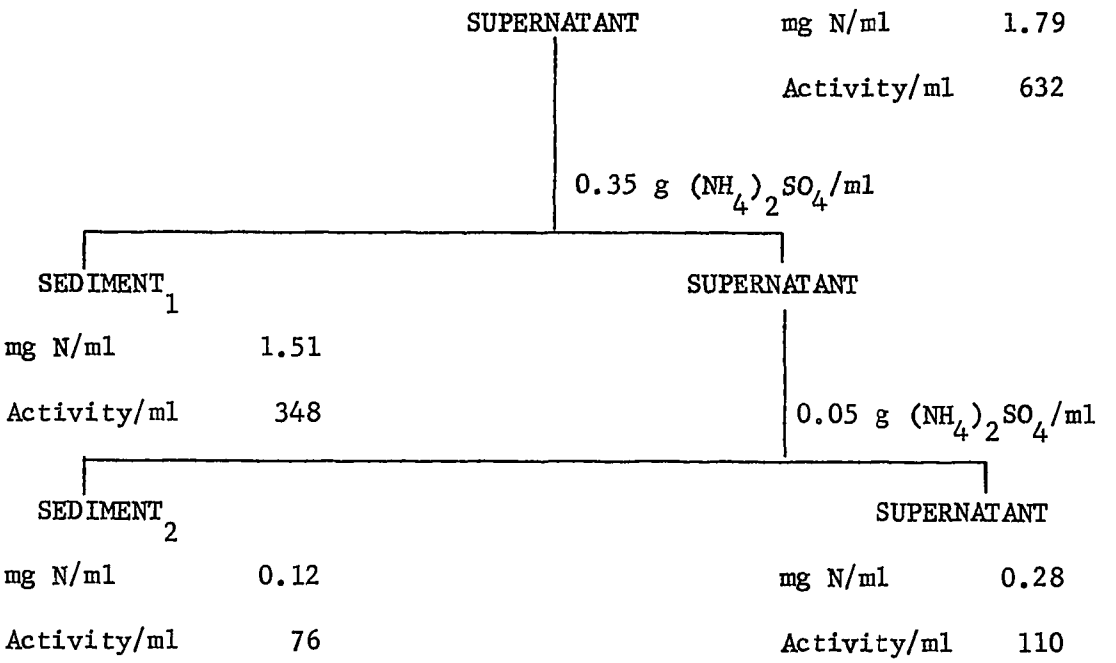
Fraction	Activity Units	Nitrogen mg/ml
$Sediment_1$	168	1.10
$Sediment_2$	128	0.27
Supernatant	284	0.48
TOTAL	580	1.85
Initial Supernatant	632	1.79

A unit of activity equals a change in OD of 0.001 per minute.

FIGURE 28

AMMONIUM SULFATE FRACTIONATION

0.35 - 0.40 g $(NH_4)_2SO_4$/ml Fractionation



SUMMATION

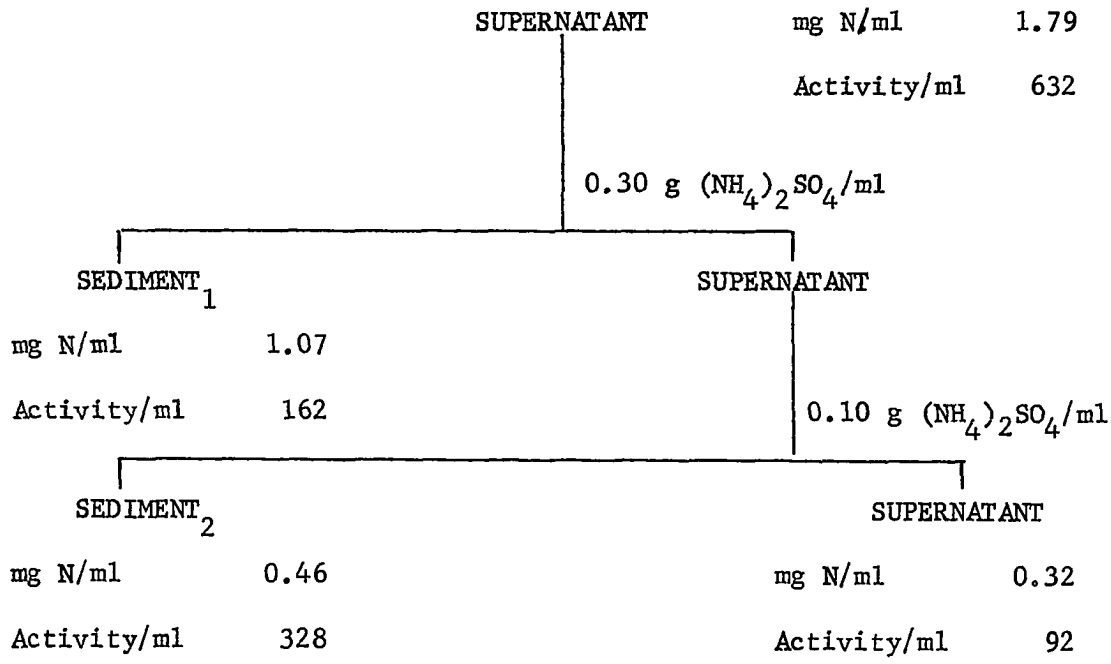
Fraction	Activity Units	Nitrogen mg/ml
$Sediment_1$	348	1.51
$Sediment_2$	76	0.12
Supernatant	110	0.28
TOTAL	534	1.91
Initial Supernatant	632	1.79

A unit equals a change in OD of 0.001 per minute.

FIGURE 29

AMMONIUM SULFATE FRACTIONATION

0.30 - 0.40 g $(NH_4)_2SO_4$/ml Fractionation



SUMMATION

Fraction	Activity Units	Nitrogen mg/ml
Sediment$_1$	162	1.07
Sediment$_2$	328	0.46
Supernatant	92	0.32
TOTAL	582	1.85
Initial Supernatant	632	1.79

A unit of activity equals a change in OD of 0.001 per minute.

Fractionation on DEAC Column

Fractionation of 43-55 Per Cent Saturation $(NH_4)_2SO_4$ Fraction on DEAC Column

Procedure. The fraction which represents 43-55 per cent saturation with ammonium sulfate was obtained from guinea pig liver supernatant$_3$ (25 per cent wet weight) in the following manner. The supernatant$_3$ was treated with solid ammonium sulfate at a concentration of 0.3 g/ml and allowed to stand 1 hour followed by centrifugation at 24,500 X G for 1 hour. The concentration of ammonium sulfate in this supernatant was increased to 55 per cent saturation by addition of 0.1 gr of ammonium sulfate per ml. This was again allowed to stand 1 hour followed by centrifugation at 24,500 X G for 1 hour. The supernatant from this centrifugation was discarded and the sediment was dissolved in 5 ml of 0.005 M potassium phosphate buffer pH 7.0. This solution was then dialyzed against a continuous change of 0.2 M potassium phosphate buffer pH 7.0 until a negative ninhydrin test was given by the dialyzing medium. This time was approximately 18 hours. The solution was then dialyzed against 0.005 M potassium phosphate buffer pH 7.0 for 72 hours. The volume of the solution had increased to 10 ml at this point, 8 of which were placed on the N,N-diethylaminocellulose (DEAC) column.

The activity of the material applied was 35.0×10^2 units/ml and contained 5.8 mg N/ml. The activity would then be 6.03×10^2 units/mg N and, since 8 ml were applied to the column, 280×10^2 units of activity and 46.4 mg of nitrogen were applied to the column.

The DEAC column fractionation procedure was patterned after that

applied to serum by Sober and Peterson (56). The column was prepared in the following way. Twenty-eight grams of DEAC were washed once with approximately 200 ml of 0.005 M sodium phosphate buffer pH 7.0. The pH of the eluate which was decanted off from the suspension was 8.6. The DEAC was then washed two more times with 200 ml of 0.02 M sodium phosphate buffer pH 4.6. The pH of the first and second eluates which were decanted off was 8.5 and 7.2 respectively. The DEAC was then allowed to stand 18 hours with 0.005 M sodium phosphate buffer pH 7.0. It was necessary to remove a portion of the very small particles of DEAC by repeated washing with buffer, centrifugation at low speed, and decantation to prevent the sintered glass filter at the bottom of the column from being plugged.

The DEAC thus prepared was made into a slurry in the 0.005 M sodium phosphate buffer pH 7.0 and poured into a 40 x 2.5 cm column with sintered glass filter at the bottom. The column was allowed to settle by gravity overnight with a constant flow of buffer to prevent drying of the column. The column was then packed by applying 15 lb pressure/sq. in., after which the column was equilibrated for 12 hours with 0.005 M sodium phosphate buffer pH 7.0.

Eight ml of the tissue fraction described above were then pipetted on to the top of the column and allowed to flow down by gravity until the level of the solution reached the top of the DEAC. The column was then covered with 0.005 M sodium phosphate buffer and 15 lbs. of pressure again applied. The pressure was applied to a reservoir bottle which, in turn, was connected to a 250 ml mixing flask over a magnetic stirrer and then to the column. Thus, a change of concentration of

constituent in the reservoir would gradually change before becoming an eluate for the column.

The flow rate was approximately 40 ml/hr and approximately 10 ml fractions were collected with a fraction collector. The temperature of the room in which the column was run was 17°C.

In Table 29 is given the change of buffer pattern which was followed during the course of the development of the column.

TABLE 29

CHANGE IN BUFFER PATTERN

Beginning buffer 0.005 M sodium phosphate pH 7.0							
Change Made:							
Fraction 30	0.02 M	sodium	phosphate	buffer	pH	6.0	
" 87	0.05 M	"	"	"	"	"	
" 113	0.05 M	"	"	"	"	4.3	
" 115	0.05 M	"	"	"	"	"	plus 0.1 M NaCl
" 168	0.1 M	"	"	"	"	3.9	plus 0.5 M NaCl
" 242	1.0 M	NaOH					

The optical density at 2800 Å of each tube (fraction) was determined and those which showed the presence of protein by absorption at this wave length were tested for enzyme activity using cofactor TPN and substrate testosterone.

Results. These results, including pH of each fraction and the

optical density of each fraction for the entire separation, are shown in Figure 30. The portion having enzyme activity is compared to OD 2800 Å in Figure 31.

Fractions were pooled, as shown in Table 30, and the nitrogen content of each of the pools was determined. The total measured volume of each pool is given and the total enzyme activity as a summation of the individual fractions of pool 2 is also given.

It will be noted that the protein fraction which contained enzyme activity came off the column quickly in that it was contained in fractions 11-16. Despite this fact, only about 5 per cent of the total activity was recovered from the column (14.35×10^2 units recovered and 280×10^2 applied). Seventy-eight per cent of the total nitrogen was recovered in that 36.3 mg N were recovered from 46.4 mg N applied.

It was felt that the rate of the flow was so slow that much of the enzyme was denatured on the column. A portion of the enzyme solution which was not applied to the column was allowed to stand in the room where the fractionation was carried out. In this solution the units/ml were decreased from 3.5×10^3 to 2.3×10^3, which is a definite drop in activity but definitely not of the order of that put on the column. Later columns were constructed shorter in length and the flow rate was increased. It will be seen that nearly 100 per cent of the activity could be recovered (page 112) from these columns.

Comparison of Supernatant and Ammonium Sulfate Fraction Fractionated on DEAC Column

Procedure. The 43 to 55 per cent saturation with $(NH_4)_2SO_4$ fraction was obtained from 40 ml of guinea pig liver supernatant 105,400 X G.

FIGURE 30

FRACTIONATION ON DEAC COLUMN

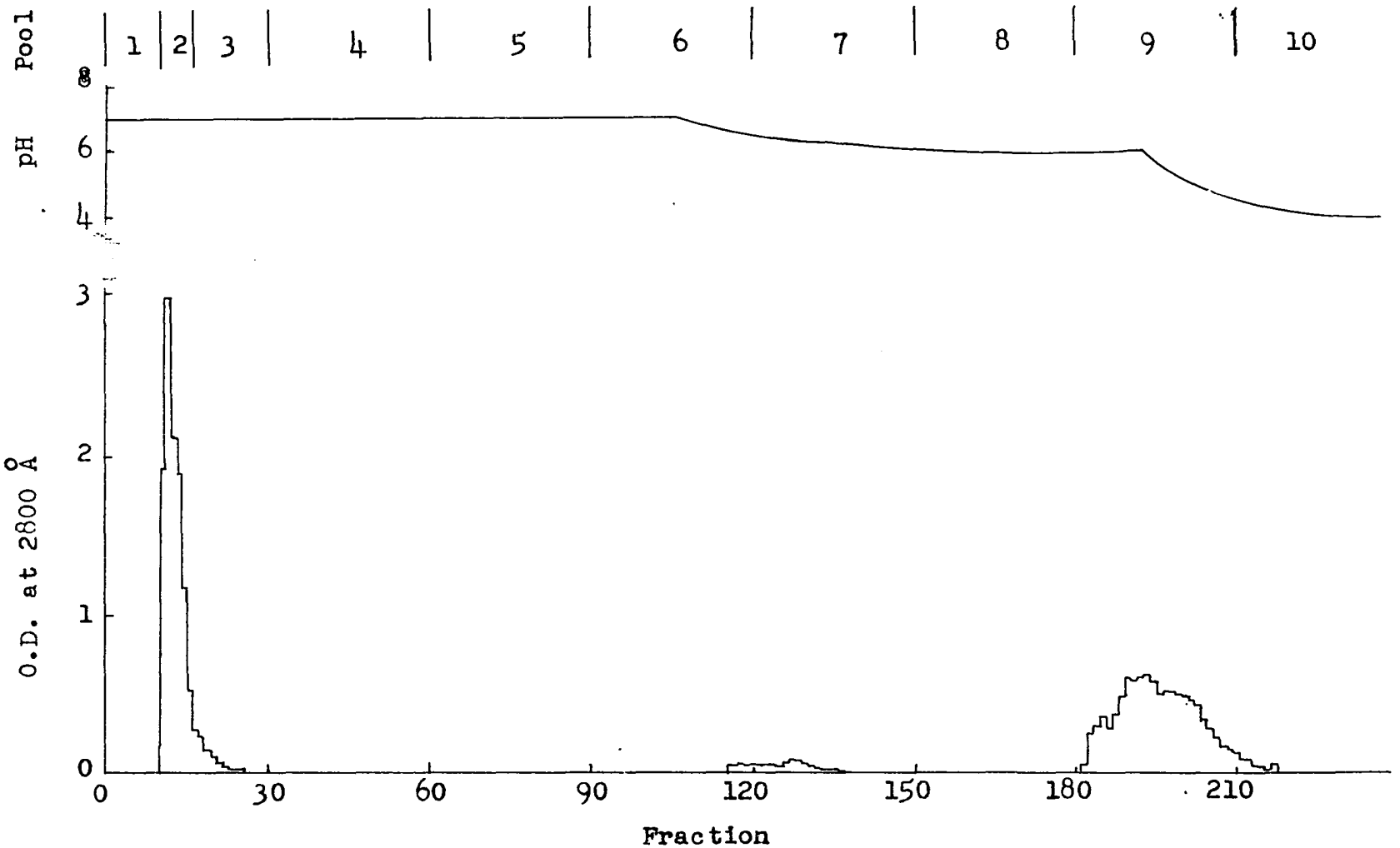


FIGURE 31

CORRELATION OF ENZYME ACTIVITY WITH O.D.$_{2800\text{Å}}$ FROM DEAC COLUMN

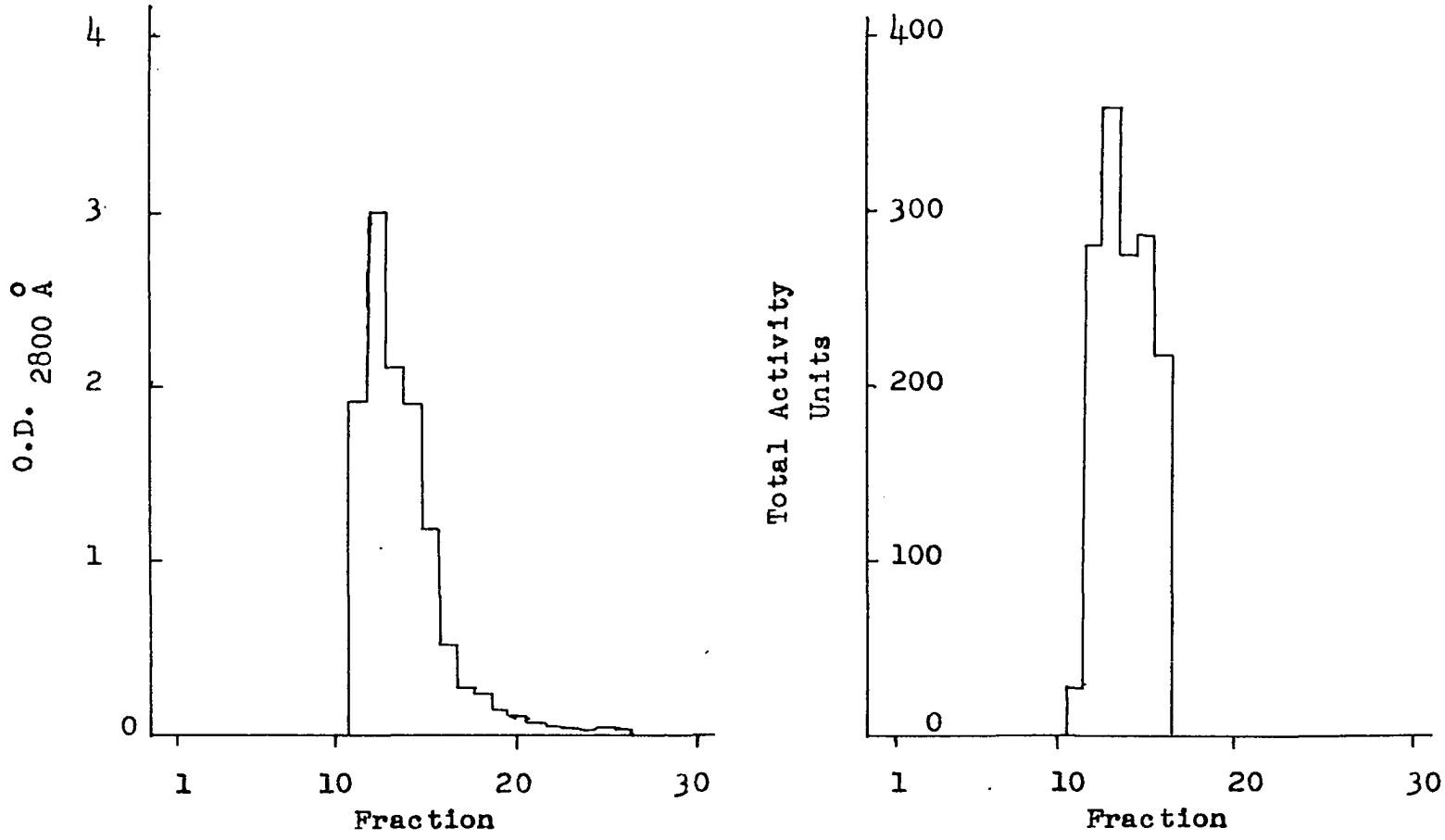


TABLE 30

FRACTIONATION ON DEAC COLUMN

Pool #	Fractions	Total ml	mg N per 50 ml	Total N	Enzyme Activity Units per mg N	Total Activity Units
1	1-10	100	0.27	0.54		
2	11-16	52	13.50	14.04	102	1435
3	17-30	123	0.95	2.34		
4	31-60	239	0.54	2.58		
5	61-90	193	0.37	1.42		
6	91-120	228	0.41	1.87		
7	121-150	300	0.52	3.12		
8	151-180	297	0.43	2.56		
9	181-210	402	0.43	3.45		
10	211-242	414	0.11	0.90		
11	NaOH fract.	1030	0.17	3.50		
Total				36.32		1435

The sediment was dissolved in 1.5 ml of 0.005 M sodium phosphate buffer pH 7.0 and dialyzed against 0.005 M sodium phosphate buffer pH 7.0 until a negative ninhydrin test was given by dialyzing medium. The total volume after dialysis was 4.6 ml.

This total amount (4.6 ml) was added to approximately 3 g DEAC which had been previously equilibrated with 0.005 M buffer pH 7.0 and more buffer (20.4 ml) was added to give a smooth slurry and a total volume of 25 ml.

To another 3 grams of DEAC prepared as above, 25 ml of supernatant 105,400 X G were added and a slurry made.

These two slurries were allowed to stand in the cold for 1/2 hour, after which time they were poured onto respective 5 cm Buchner funnels and the eluate was collected from each in fractions of about 10 ml as it dripped through by gravity. As each fraction was collected an equal amount of 0.005 M buffer pH 7.0 was added to the top of the Buchner.

Enzyme activity was determined by the usual test system (cofactor TPN) and nitrogen content was also determined by the Kjeldahl method.

Results. The results are tabulated in Tables 31 and 32. First, it will be noted that nearly 100 per cent of the enzyme activity was recovered from the supernatant applied to the column. The nitrogen assay was lost on fraction 3 but, with an estimation of nitrogen for the fraction, about 60 per cent of the original nitrogen was contained in the fraction removed containing enzyme. This represents about 1.6 fold purification by addition of supernatant directly to DEAC column, but with

TABLE 31

FRACTIONATION OF SUPERNATANT 105,400 X G ON DEAC COLUMN

Fraction	Activity[a] per ml Units	ml	Total Activity[a] 10^2 Units	mg N per ml	Total N	Activity[a] per mg N Units
1	1170	15.0	176	1.70	25.5	687
2	920	10.5	97	1.70	17.9	544
3	230	11.0	25		(5.0)[b]	
4	40	11.0	4	0.07	1.0	409
Total			302		49.4	616[c]
Supernatant	1230	25.0	308	3.28	82.0	376

a. A unit of activity equals a change in OD of 0.001/min.

b. Estimated.

c. Calculated from totals.

TABLE 32

FRACTIONATION OF AMMONIUM SULFATE FRACTION (43-55 PER CENT SATURATION) ON DEAC COLUMN

Fraction	Activity[a] per ml Units	ml	Total Activity[a] 10^2 Units	mg N per ml	Total N	Activity[a] per mg N Units
1	630	15.0	95	0.56	8.4	1121
2	480	10.5	50	0.48	5.0	1008
3	300	11.0	33	0.21	2.3	1429
4	180	11.0	20	0.07	0.8	2500
5	50	10.0	5	0.18	0.2	3000
6	50	10.2	5	0.01	0.1	600
Total			208		16.8	1240[b]
Supernatant	1230	40.0	493	3.28	131.2	376

a. A unit of activity equals a change in OD of 0.001 per minute.

b. Calculated from totals.

nearly 100 per cent recovery of the enzyme activity.

Fractionation of supernatant 105,400 X G first to obtain the 43-55 per cent of ammonium sulfate fraction followed by application of this fraction to DEAC results in the loss of approximately 60 per cent of the total enzyme activity and 93 per cent of the total nitrogen, a 3.3 fold purification, which is twice that obtained by applying the supernatant directly to the DEAC column.

Purification by Successive Alternate Precipitation with Ammonium Sulfate and Separation on DEAC Column

Procedure. One hundred twelve grams of guinea pig liver were homogenized in 450 ml of 0.25 M sucrose. This homogenate was centrifuged at 2,000 RPM (900 X G) for 45 minutes. The supernatant was centrifuged in the Spinco Model L centrifuge at 20,000 RPM (40,000 X G) for 1 hour. Ninety six grams of ammonium sulfate were added to 350 ml of this supernatant (0.275 g/ml) and allowed to stand at $4^{o}C$ for 1 hour, followed by centrifugation at 25,000 X G for 1/2 hour. The sediment was discarded. One 100 ml tube was accidentally broken during centrifugation. To the 250 ml of remaining supernatant 32 grams of ammonium sulfate were added (to bring the concentration to 0.4 g/ml) and allowed to stand for 1 hour at $4^{o}C$, followed by centrifugation at 25,000 X G for 15 minutes. The sediment was dissolved in 10 ml of 0.005 M sodium phosphate buffer pH 7.0 and dialyzed against a continual flow of buffer of the same concentration overnight. After dialysis the total volume was 28 ml. Twenty five ml were put with approximately 10 g (damp weight) DEAC to make a slurry which was put on a DEAC column (gravity packed) which was 2.5 cm

in diameter and 10 cm in height and then eluted with 0.005 M sodium phosphate buffer pH 7.0.

The fractions from this first column (column 1) which exhibited enzyme activity were pooled (total 60 ml), and 50 ml of this pool were treated with 20 g ammonium sulfate for 30 minutes, followed by centrifugation at 24,500 X G for 30 minutes. The sediment was dissolved in 0.005 M buffer and dialyzed against 0.005 M buffer overnight. The total volume after dialysis was 14.5 ml, 12.5 of which were placed on a second DEAC column (column 2), similar to the first column.

The enzyme activities and the $OD_{2800\ \text{Å}}$ were determined on fractions and pools from the columns.

Results. The results are given in Table 33. The results show a five-fold purification from the homogenate on the basis of activity/mg N, but only 17 per cent of the enzyme activity was retained. This recovery may be increased somewhat considering that the total amount of tissue was not carried to each subsequent step since it was used for the determination of enzyme activity and absorption at 2800 Å.

The degree of purification on the basis of activity/$OD_{2800\ \text{Å}}$ is not increased by the second ammonium sulfate precipitation and subsequent fractionation on the DEAC column if one considers the fractions from each column which contained the most enzyme activity. That would be a comparison of fraction 3 column 1 which had an activity of 1.02 X 10^2 units/OD 2800 Å with fraction 3 of column 2 which had an activity of 0.85 X 10^2 units/$OD_{2800\ \text{Å}}$. Thus, it would seem that further purification could not be obtained by these methods.

TABLE 33

PURIFICATION BY SUCCESSIVE ALTERNATE PRECIPITATION WITH AMMONIUM SULFATE AND SEPARATION ON DEAC COLUMN

Fraction	mg N per ml	ml	Activity per ml 10^2 Units	Total Activity 10^2 Units	OD 2800 Å	Activity per $OD_{2800\ Å}$ 10^2 Units	Activity per mg N 10^2 Units
Homogenate	6.26	250	9.50	2375	133.0	0.071	1.52
Ammonium Sulfate ppt.		28	32.75	918	77.8	0.42	
put on col. 1		(25)		(820)			
Column 1 Fractions							
1		10	0.12	1.2	0.9	0.13	
2		15	19.50	293	30.2	0.64	
3		15	21.75	326	21.4	1.02	
4		15	9.20	138	10.9	0.84	
5		15	5.70	86	5.9	0.97	
6		--	0.87	---	1.5	0.58	
Pool 1 Fractions 2,3,4,5		60 (50)	14.00	840 (655)			

Numbers in parenthesis indicate amounts carried forward to next step.

TABLE 33 continued

Fraction	mg N per ml	ml	Activity per ml 10^2 Units	Total Activity 10^2 Units	OD 2800 Å	Activity per $OD_{2800\ Å}$ 10^2 Units	Activity per mg N 10^2 Units
Ammonium Sulfate ppt		14.5	33.75	489	46.9	0.72	
put on col. 2		(12.5)		(42)			
Column 2 Fractions							
1		15	0	0	0.05	0	
2		12	0.14	1.7	0.06	2.37	
3		30	8.85	265.0	10.50	0.85	
4		15	5.7	85.5	3.55	1.60	
5		15	2.91	43.6	1.76	1.65	
6		15	1.53	23.0	0.54	2.89	
7		15	1.02	15.3	0.31	3.29	
Pool 2 Fractions 3,4,5,6	0.91	60	6.80	408	6.70	1.01	7.50

Activity of Lyophilized Supernatant and Lyophilized Supernatant Separated on DEAC Column

Lyophilization of supernatant 105,400 X G was carried out in order to determine if this process had any denaturing effect on the enzyme in this fraction. This was done so that any fractions purified by any means might be preserved by this method.

Procedure. Fifteen ml of supernatant (105,400 X G for 2 hours) from guinea pig liver homogenized in water at 25 per cent wet weight were lyophilized. The enzyme activity (TPN) of this preparation dissolved in 15 ml of water was compared with the enzyme activity of the original supernatant. The lyophilized powder was readily dissolved in water with no residue remaining.

Results. The results of lyophilization on enzyme activity are shown in Table 34. The results show that lyophilization of this fraction had no denaturing effect since the enzyme activity remained the same.

TABLE 34

Fraction	Mg N/ml	Act/ml Units	Act/mg N Units
Homogenate	6.17	830	135
Supernatant	4.14	840	203
Lyophilized supernatant redissolved in water	3.98	850	213

One experiment was run to determine if this lyophilized powder might fractionate better on DEAC column to give a better purification

since it could be applied in a more concentrated form to the column.

Procedure. Guinea pig liver was homogenized at 25 per cent wet weight in water in the cold in the Virtis by the usual procedure. The homogenate was centrifuged at 105,400 X G for 2 hours and the sediment was discarded. Forty five ml of the supernatant 105,400 X G were lyophilized and the powder was applied in dry form to a DEAC column 2.5 cm in diameter and 25 cm in height (gravity packed) and enough 0.005 M sodium phosphate buffer pH 7.0 (approximately 10 ml) was added to the top of the column to solubilize the powder and deposit it on the top layer of the column. Elution was then continued with 0.005 M sodium phosphate buffer pH 7.0. The first eluate coming off the column which exhibited absorption at 2800 Å was collected and lyophilized, resulting in a total of 1.464 g of powder. The enzyme activity and nitrogen content of the homogenate, supernatant and lyophilized material made up at a concentration of 3 mg/ml were determined.

Results. The results are given in Table 35. The purification was only two fold on the basis of activity/mg N but recovery was 92 per cent, again indicating that little or no inactivation is caused by lyophilization.

Use of Preceding Methods for Preparation of Enzyme Fraction for Future Use

It appeared at this point that the best method of those tried for the purification of the TPN enzyme component was by ammonium sulfate fractionation of supernatant followed by fractionation of this fraction on a DEAC column. Two preparations were made by this method and lyophilized for the purpose of further characterization.

TABLE 35

DEAC COLUMN FRACTIONATION OF LYOPHILIZED SUPERNATANT

Fraction	Mg N/ml	Total N	Act/ml Units	Total Units	Act/mg N Units
Homogenate 25%	6.05		1090		180
Supernatant (105,400 X G)	3.78	170	1070	481	283
Lyophilized fraction from column (conc. 3 mg/ml)	0.25	122[a]	92	449[b]	368

a. Calculated from Mg N/mg powder 0.083 X 1,464 = 122.

b. Calculated from Act/mg powder being 0.307 X 1,464 = 449.

Procedure. The 43-55 per cent of saturation with ammonium sulfate fraction was obtained from guinea pig liver supernatant (105,400 X G) by the same process described previously on page 102 of this chapter. The fraction was dissolved in 0.005 M sodium phosphate buffer and dialyzed against a continuous change of 0.005 M phosphate buffer pH 7.0 for 18 hours. The dialysate was placed on a 2.5 cm diameter, 15 cm in height DEAC column, and elution was carried out with buffer of the same concentration. The first eluate which had absorption at $_{2800}$ Å was lyophilized and solutions of the lyophilized powder were analyzed for enzyme activity (TPN) and nitrogen content.

Results. The results of these two preparations are summarized in Table 36. The five fold purification shown before (Table 33) was given in the first preparation while in the second the purification was

TABLE 36

SUMMARY OF PREPS. 36 AND 37

Fraction	mg N per ml	ml	Activity* Units per ml	Total Activity 10^2 Units	Activity* Units per mg N
		Prep. 36			
Homogenate	6.68		875		131
Supernatant	3.88	100	895	895	231
Lyophilized fraction from column (3 mg/ml)	0.25	42	167	70	676
	Recovered 125 mg of dry white material				
		Prep. 37			
Supernatant	3.65	100	954	954	261
Lyophilized fraction from column (3 mg/ml)	0.26	100	97	107	373
	Recovered 329 mg of dry white material				

* A unit of activity equals a change in OD of 0.001 per minute.

not as good. These two fractions were used later for some further characterization in Chapter VI.

CHAPTER VI

FURTHER CHARACTERIZATION OF ENZYME ACTIVITIES

Activity on Various Substrates

Procedure. The activities of various liver fractions were determined on eight different steroids. The initial experiment was carried out with homogenate, sediment 24,500 X G, and supernatant 24,500 X G as the enzyme sources and either DPN or TPN as the cofactor. The steroids were added as water solutions having been prepared as described in Chapter II. The reaction mixture was as follows: 1.0 ml of 0.18 M sodium pyrophosphate buffer pH 9.55, 0.2 ml DPN (15 mg/ml) or TPN (5 mg/ml), 1.8 ml of the steroid solution, and tissue as indicated below. The reactions were blanked against an identical reaction mixture except that 1.8 ml of water were substituted for the steroid solution. The reaction rate was again measured in the Cary at 3400 Å. The following steroids were used as substrates: Testosterone, etiocholan-17 β -ol-3-one, etiocholan-3α-ol-17-one, dehydroepiandrosterone, epiandrosterone, epitestosterone, androsterone and androstan-17 β -ol-3-one. The structure of these eight steroids are illustrated in Chapter I.

Results. In Table 37 are given the results of the experiments in which DPN was used as the cofactor. The source of enzyme used in

TABLE 37

THE OXIDATION OF VARIOUS STEROIDS BY GUINEA PIG LIVER FRACTIONS

Substrate	Conc. µg per 3 ml reaction	Units Activity* per ml of 10% Prep.					
		DPN			TPN		
		Homog.	Sed.$_2$	Super.$_2$	Homog.	Sed.$_2$	Super.$_2$
Testosterone	57	1513	1440	213	540	12	616
Androstan-17 β -ol-3-one	11	953	980	227	140	0	148
Etiocholan-17 β -ol-3-one	70	887	467	266	1044	0	888
Etiocholan-3 α -ol-17-one	45	320	80	140	84	16	96
Dehydroepiandrosterone	50	120	13	86	20	0	4
Epiandrosterone	27	180	20	147	0	0	12
Epitestosterone	30	247	53	213	0	0	24
Androsterone	11	227	53	160	136	0	100

* A unit of activity equals a change in OD of 0.001 per minute.

this experiment was 0.03 ml of 5 per cent liver. The homogenate is active to a varying degree on all eight of these substrates, but a comparison can hardly be made between rates because the concentration of steroid per reaction is not the same in any case due to the differences in the solubility of the various steroids in water. This means that the reaction was not necessarily zero order with respect to steroid in every case.

Disregarding the effect the concentration of steroid may have on rate, we may say that the sediment or mitochondrial fraction is active only on testosterone, etiocholan-17 β -ol-3-one, and androstan-17 β -ol-3-one with only small and questionable activity on the other five steroids. The supernatant from 24,500 X G has small but seemingly nearly equal activity on all these substrates when DPN is used as cofactor.

The results using TPN with the various substrates and various liver fractions are given in Table 37. Liver (0.05 ml of 5 per cent) was used in these experiments. The homogenate shows the greatest activity on etiocholan-17 β -ol-3-one, which is approximately two times the activity on testosterone. It should be noted that the concentration of etiocholan-17 β -ol-3-one is higher than testosterone, which may or may not have had some bearing here since testosterone is not at optimum concentration (Figure 22). There is a moderate degree of activity on etiocholan-3 α -ol-17-one, androsterone and androstan-17 β -ol-3-one, with no activity on the other three steroids. The mitochondrial fraction shows no activity on any of the steroids with TPN as the cofactor. The activity of the supernatant on these steroids parallels

that of the homogenate with TPN.

Activity Using Higher Concentrations of Steroid

Procedure. The activities of two liver fractions on these same eight steroids were investigated more extensively using a system in which the concentration of steroid could be increased further. The sediment 24,500 X G (mitochondria) from preparation number 36 was lyophilized and suspensions of this powder were used as the enzyme source when DPN was used and the lyophilized powder from preparations numbers 36 and 37 was used as the enzyme source when TPN was used as the cofactor. These powders were made up in water at a concentration of 6 mg/ml and 3 mg/ml for the mitochondrial and purified preparations from preparations 36 and 37 respectively. Five-hundredths ml of these solutions were used per reaction.

The substrates were prepared as alcohol solutions at such a concentration that when 0.05 ml of the solution was added to the reaction mixture the final concentration would be from 5 to 300 µg/reaction. However, the only available source of etiocholan-17 β-ol-3-one at the time of the experiment was a water solution. Therefore, the concentration of it could only be varied between 5 and 90 µg/reaction.

The following test system was used: 1.0 ml of sodium pyrophosphate buffer 0.18 M pH 9.55, 0.2 ml of DPN (15 mg/ml) or TPN (5 mg/ml), 0.05 ml of substrate, water to make 3 ml and 0.05 ml of enzyme solution. Alcohol (0.05 ml) was contained in the blank in addition to all the other reactants with the exception of the steroid. Thus, any alcohol dehydrogenase present was automatically subtracted from the reaction.

Results. In Tables 38 and 39 are given the results of these experiments. Testosterone and etiocholane-17β-ol-3-one are the only two substrates which demonstrate reasonable activity, and this activity is manifested with both the mitochondrial fraction with DPN and the purified supernatant fraction with TPN. Androstan 17β-ol-3-one is also active with both fractions, but because of the low solubility of this steroid in water (6.2 µg/ml) the steroid would precipitate out at concentrations higher than 70 µg/reaction, making rate measurement impossible by the method used. Androsterone also exhibits some activity at higher concentrations and behaves entirely differently from the other active steroids in that no activity was noted at low concentrations and then a sharp rise in activity was noted in the range of substrate concentration from 100 to 300 µg/reaction. No activity was noted on this substrate with the TPN system using the lyophilized powder of purified supernatant 105,400 X G.

Michaelis-Menten Constants

Procedure. Michaelis-Menten constants (58) were determined by the method of Lineweaver and Burk (59) from data on the two substrates that gave good activity without the complication of insolubility, etc., i.e. testosterone and etiocholane-17β-ol-3-one.

Both water and alcohol solutions of testosterone were used but only a water solution of etiocholane-17β-ol-3-one was used. The alcohol solutions of testosterone were made at such concentrations that addition of 0.05 ml would result in the addition of 5-300 µg of testosterone/reaction, and the water solution was analyzed to be of the concentration

TABLE 38

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF THE DPN-LINKED OXIDATION OF VARIOUS SUBSTRATES BY GUINEA PIG LIVER MITOCHONDRIA

Substrate conc. μg per 3 ml reaction	Activity* in Units				
	Testosterone	Androstan-17 β-ol-3-one	Dehydroepi-Androsterone	Androsterone	Epi-androsterone
5	5.5	4.0	0.4	0	0.4
10	8.8	4.2	0	0.2	0
20	12.8	6.8	0	0.3	0
30	10.8	6.6	0		0.1
40	12.4	8.7	0.6		0.3
50	13.7	9.6	0.3	0.6	0.1
60	13.3	7.1			
70	15.5	6.6			
80	12.0				
90	11.3				
100	14.3	Substrate precipitated	0	1.0	0.9
150	14.9		0.1	3.7	1.7
200	12.6		0	7.5	Non-linear curves
250	12.6		0	12.7	
300	12.0		0	11.2	

* A unit of activity equals a change in OD of 0.001 per minute.

TABLE 38 continued

Activity* in Units		Substrate conc. µg per 3 ml reaction	Activity* in Units Mitochondria	Activity* in Units Homogenate
Etiocholan-3 ∝ -ol-17-one	Epi-testosterone		Etiocholan-17 β -ol-3-one	Etiocholan-17 β -ol-3-one
0.2	0	5	5.3	6.8
0.6	0.5	10	6.0	9.7
0.4	0.5	15	7.0	12.7
0.3	0.4	20	7.1	12.6
0.1	0.5	25	7.0	13.7
0.8	0.7	30	7.1	13.8
0	0.4			
0.6	0.7			
0	0.7			
0.2	0.2			
0.4	0.2			
0.6	0.4			
1.0	0.6			
1.5	Non-linear			
0.1				

TABLE 39

THE EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF THE TPN-LINKED OXIDATION OF VARIOUS SUBSTRATES BY GUINEA PIG LIVER PURIFIED SUPERNATANT

Substrate conc. μg per 3 ml reaction	Activity* in Units				
	Testosterone	Androstan-17 β-ol-3-one	Dehydroepi-androsterone	Androsterone	Epi-androsterone
5	1.5	1.1	0	0.1	0
10	2.7	2.2	0	0	0
20	4.1	1.8	0	0.2	0.2
30	5.3	2.1	0.1	0	0
40	6.3	3.3	0		0
50	6.7	2.3	0	0	0
60	7.3	2.5	0		0
70	6.9	2.4	0		0.3
80	7.4		0.6		0
90	7.5		0		0.2
100	7.7		0	0	0
150	7.9		0		0.3
200	8.1		0	Non-linear reaction	2.7
250	8.3		0		
300	7.7		0.1		

* A unit of activity equals a change in OD of 0.001 per minute.

TABLE 39 continued

Activity* in Units		Substrate conc.	Activity* in Units	
Etiocholan-3 α -ol-17-one	Epi-testosterone	µg per 3 ml reaction	Etiocholan-17 β -ol-3-one	Etiocholan-17 β -ol-3-one
0.1	0.5	5	7.5	7.3
0.4	0.1	10	9.9	10.9
0.3	0.7	15		11.8
0	0.5	20	15.8	15.7
0.2	0.6	25		15.2
0.1	0.9	30	19.0	16.2
	0.6	35		17.3
	0.8	40	22.5	18.4
	0	45		20.8
	0.7	50	22.7	20.5
0.2	0.4	55		20.4
0.2	1.2	60	22.2	21.1
0.4	0.9	65		22.2
0.1	Non-linear	70	23.6	21.6
0.2		75		21.8
		80	24.4	22.5
		85		22.4
		90	23.9	22.1

of 32.8 μg/ml. The etiocholane-17β-ol-3-one solution was analyzed to be of the concentration of 50 μg/ml.

Using TPN as a cofactor and testosterone as substrate the following tissue preparations were used as the enzyme source: Homogenate preparations 32 and 18, supernatant 105,400 X G preparation 18, and pool 2 from the purification of preparation 32.

Using TPN as a cofactor and etiocholane-17β-ol-3-one as the substrate the lyophilized powder from preparation 37 was used as the enzyme source.

Using DPN as cofactor and testosterone as the substrate a fresh preparation of mitochondrial fraction of guinea pig liver was used.

Using DPN as cofactor and etiocholane-17β-ol-3-one as substrate a fresh preparation of homogenate and mitochondrial fraction was used. The reaction mixtures used were described previously in Table 3.

The K_s values were calculated by the Lineweaver-Burk (59) method from a graphical representation of the data in which $\frac{1}{v}$ was plotted against $\frac{1}{s}$. The following formula is employed:

$$\frac{1}{v} = \frac{K_s}{V}\frac{1}{s} + \frac{1}{V}$$

where V = maximum velocity
v = velocity
s = Molar substrate concentration in the reaction
K_s = Michaelis-Menten constant

In plotting $\frac{1}{v}$ against $\frac{1}{s}$ it is obvious from the above formula that $\frac{K_s}{V}$ is equal to the slope of the line and $\frac{1}{V}$ is the y intercept. V and the slope may be obtained from the graphical plot of $\frac{1}{v}$ against $\frac{1}{s}$ and then K_s may be obtained by solving K_s = V x slope.

Results. Table 40 is a summary of the K_s values obtained. The values obtained with testosterone and TPN with the various preparations agree quite closely in the range of 6×10^{-5}. The value with etiocholan-17β-ol-3-one as substrate and TPN with the purified preparation 37 is approximately 1/3 of this value.

All K_s values determined with DPN as the cofactor are in the range of 10^{-6} instead of the 10^{-5} value determined with TPN. The values obtained using etiocholan-17β-ol-3-one as substrate and homogenate and mitochondria as enzyme source differ about 2 fold.

The K_s values obtained would indicate that the DPN enzyme had a greater affinity for both testosterone and etiocholan-17β-ol-3-one than did the TPN enzyme. Etiocholan-17β-ol-3-one may have a higher affinity than testosterone in the case of the TPN enzyme but the difference is not striking. From the data obtained it would be difficult to determine if either of the substrates is preferred in the case of the DPN enzyme. It should be pointed out that the DPN K_s values were all determined with quite impure preparations while those of the TPN were done with some preparations which had been purified approximately 5-fold on the basis of activity/mg N. Sweat, et al., (60) have reported an enzyme in steer liver which oxidizes testosterone to 4-androstene-3,17-dione and which requires DPN as a hydrogen acceptor. This enzyme, however, appears to be in solution in the cytoplasm. They also report a K_s of 3.3×10^{-5} mole per liter.

Effect of Activators

Procedure. Since a number of metallic ions are known to activate

TABLE 40

K_s SUMMARY

Substrate	K_s Moles/Liter
TPN	
Testosterone (alcohol solution)	
Homogenate (prep 32)	6.325×10^{-5}
Purified prep (prep 32, pool 2)	5.65×10^{-5}
Testosterone (water solution)	
Homogenate	5.60×10^{-5}
Supernatant	6.44×10^{-5}
Etiocholan-17 β -ol-3-one (water solution)	
Purified prep (Lyophilized prep 37)	2.069×10^{-5}
DPN	
Testosterone (alcohol solution)	
Mitochondria	8.348×10^{-6}
Etiocholan-17 β -ol-3-one (water solution)	
Mitochondria	2.938×10^{-6}
Homogenate	7.27×10^{-6}

specific enzyme systems, seven common activators were used to determine if any of them would have any effect on the TPN specific testosterone oxidizing enzyme of guinea pig liver. The following activators were used: Mn^{++}, Mg^{++}, Co^{++}, Ca^{++}, Al^{+++}, Zn^{++} and Cu^{++} at final concentrations ranging from 1×10^{-3} to 1×10^{-6} M. These ions were used as their chloride salts. Lyophilized purified supernatant 105,400 X G from preparation 36 was used as the enzyme source, and it was made up at the concentration of 3 mg/ml in water. The standard reaction mixture described on page 19 using TPN as cofactor and water solution of testosterone as substrate was used. One-tenth ml of the activator solution was added, so the total volume for this experiment was 3.1 ml/cuvette plus enzyme.

Results. In Table 41 are given the results of the experiment. No activation of the enzyme was noted with any of the ions used. Some indication of inhibition was noted with Co^{++}, but the decrease of approximately 20 per cent may not be significant.

TABLE 41

THE EFFECT OF METALLIC IONS ON THE RATE OF THE TPN-LINKED OXIDATION OF TESTOSTERONE

Metallic Ions Final Concentration Molar	Units Activity*/mg N						
	$MnCl_2$	$MgCl_2$	$CoCl_2$	$CaCl_2$	$AlCl_3$	$ZnCl_2$	$CuCl_2$
0	620	620	539	506	588	563	588
1×10^{-6}	637	637	514	588	555	555	563
1×10^{-5}	612	629	539	555	571	563	531
1×10^{-4}	661	588	392	571	571	588	514
1×10^{-3}	588	604	433	531	588	588	457

* A unit of activity equals a change in OD of 0.001 per minute.

CHAPTER VII

SUMMARY

Homogenates of guinea pig liver are capable of using either DPN or TPN as the hydrogen acceptor while oxidizing testosterone to 4-androstene-3,17-dione. The rate of oxidation when DPN is used as hydrogen acceptor is approximately twice that when TPN is used. These two activities in guinea pig liver homogenate were separated by centrifugation showing that the DPN activity was associated with the mitochondrial fraction while the TPN activity was associated with the soluble fraction after centrifugation at 105,400 X G.

Examination of the reaction mixtures using either TPN or DPN as coenzyme and guinea pig liver homogenate and fractions as enzyme sources shows 4-androstene-3,17-dione to be the only product formed from testosterone. The rate of formation of DPNH as determined spectrophotometrically was shown to correlate with the disappearance of testosterone and appearance of 4-androstene-3,17-dione. Some indication of a complex with testosterone was given by this experiment.

The rate of oxidation of testosterone by guinea pig liver homogenates and fractions thereof with appropriate coenzyme (DPN or TPN) was studied under the influence of varying hydrogen ion concentration, pyrophosphate buffer concentration, coenzyme concentration and substrate

concentration. The results indicated the following optimum conditions and led to the formulation of the following 3 ml assay when DPN was used as coenzyme: 0.18 mMoles sodium pyrophosphate buffer (pH 9.6) to maintain the reaction at pH 9.50, 3000 μg DPN, and 60 μg testosterone. When TPN was used as coenzyme the following assay was formulated: 0.18 mMoles sodium pyrophosphate buffer (pH 9.55) to maintain the reaction at pH 9.50, 1500 μg TPN and 60 μg testosterone. This concentration of testosterone was used in assay methods for TPN activity but a concentration of testosterone which yielded maximum rates was not reached in substrate concentration experiments in which TPN was used as coenzyme.

The differences of behaviour of the two systems (DPN or TPN) to the factors above indicate that two different enzymes, one specific for DPN and one for TPN, are associated with the oxidation of testosterone in guinea pig liver. This is proposed and discussed despite the presence of small amounts of transhydrogenase activity in the homogenate and mitochondrial fraction.

A five-fold purification of the TPN active component in the soluble fraction of guinea pig liver homogenate was achieved by ammonium sulfate fractionation followed by separation on a column of N, N-diethylaminocellulose. The activity of the soluble fraction was not reduced by lyophilization.

The activity of the two systems (DPN and TPN) was determined on seven steroids other than testosterone. Activity was noted only on the 17β-hydroxy compounds. K_s values for testosterone and etiocholan-17β-ol-3-one were determined in both systems.

Seven metal ions had no activating effect when tested with the TPN system using a purified supernatant fraction as the enzyme source.

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